GENETIC AND EVOLUTIONARY CHARACTERIZATION OF PLANT PATHOGENIC XANTHOMONADS BASED ON DNA SEQUENCES RELATED TO THE *hrp* GENES

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1994

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to Dr Robert Stall for his constant support and encouragement during the course of this doctoral program. His vast expertise and willing to offer all necessary support were invaluable. I would also like to thank the other members who served on my supervisory committee, Dr Jeffry Jones, Dr James Preston, and Dr Daryl Pring, for their support and suggestions I am indebted to Dr Jerry Bartz for reviewing this dissertation.

Appreciation is extended to the Instituto Agronômico do Paraná (IAPAR), which gave permission for this study leave and provided financial support, and to the Conselho Nacional de Pesquisa (CNPq), which has granted a scholarship for this graduate program.

I am indebted to Dr Ulla Bonas of the Institut des Sciences Végétales, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France She provided the DNA sequences of the *hrp* genes that were the keystone of this work. Appreciation is extended to Gail Somodi Agricultural Research and Education Center in Bradenton, FL, who performed the ELISA tests. Jay Harrison, IFAS consulting Division of the Department of Statistics, was very helpful with the SAS programming

Special thanks are given to Jerry Minsavage for his invaluable technical assistance and friendship and to Cheri Hodge for the fatty acid analysis and her friendship

Finally I do not have enough words to thank my family Claudia, my wife, and our two sons, Ruy and Rafael. They have provided unlimited understanding and support during these years of graduate studies. They have shared their love, their dreams, and

their lives. I am also indebted to my own parents who have offered whatever support was needed

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Abstract of Dissertation Presented to the Graduate School of the University of Florida
in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

GENETIC AND EVOLUTIONARY CHARACTERIZATION OF PLANT PATHOGENIC XANTHOMONADS BASED ON DNA SEQUENCES RELATED TO THE *hrp* GENES

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August 1994

Chairman: Robert E. Stall
Major Department: Plant Pathology

Three pairs of oligonucleotide primers specific for different regions of the hypersensitive reaction and pathogenicity (*hrp*) genes of *Xanthomonas campestris* pv. vesicatoria were designed and used for amplification of homologous DNA sequences from *X. fragariae* and from 28 pathovars of *X. campestris*. No amplification occurred with genomic DNA from plant pathogenic strains of *X. campestris* pv. secalis, *X. campestris* pv. translucens, and *X. albilineans*, from nonpathogenic xanthomonads, or from plant pathogenic strains of the genera *Acidovorax*, *Agrobacterium*, *Clavibacter*, *Erwinia*, *Pseudomonas*, and *Xylella*. DNA fragments amplified with a particular primer pair were of identical size from each of the different plant pathogenic xanthomonads. However, the restriction analysis of these fragments by using frequent-cutting endonucleases revealed variation in the banding pattern of these *hrp*-related fragments. The banding patterns allowed distinction of the strains representing a pathovar or species of plant pathogenic xanthomonads.

The population structure of some taxa, i.e. *X. fragariae*, *X. campestris* pv. begoniae, *X. campestris* pv. campestris, *X. campestris* pv. malvacearum, and *X. campestris* pv. pelargonii, was homogeneous. However, the majority of the pathovars of *X. campestris* presented a high degree of sequence variability in the *hrp*-related fragments for the strains within pathovars. For instance, the citrus pathogens *X. campestris* pv. citri strains in groups A, B, and C, which cause citrus canker A, B, and C, respectively, and *X. campestris* pv. citrumelo strains in the highly aggressive group, which cause the citrus bacterial spot, had a characteristic and homogeneous restriction banding pattern within each group. In contrast, restriction fragment polymorphism was evident among strains of the moderately and weakly aggressive groups of *X. campestris* pv. citrumelo.

The phylogenetic analysis of the *hrp* genes revealed a diverse evolutionary relationship for this region of the bacterial genome of the xanthomonads. Whereas the hypothesis of coevolution of the *hrp* region with the rest of the genome from a common ancestor is supported, there is also evidence of horizontal movement of the *hrp* genes between the different plant pathogenic xanthomonads. Nevertheless, the results obtained do not substantiate the existence of genetic and functional selection pressure regarding the *hrp* genes within the xanthomonads. One of the major findings of this study is the divergence between *hrp* gene sequence and host specificity, which indicates that factors other than the *hrp* genes are likely to be involved in host speciation.

Strains of plant pathogenic xanthomonads could be detected by amplification of *hrp*-related DNA sequences from washings of pepper and tomato seeds. The diversity of the xanthomonads found on seeds assessed on the basis of restriction analysis of *hrp*-related sequences agreed very closely with the population structure determined by using other methods. The restriction banding profiles generated for the *hrp* fragments may be an easy and a discriminating approach for identification of plant pathogenic xanthomonads

CHAPTER I
INTRODUCTION

The genus *Xanthomonas* Dowson 1939 includes Gram-negative and usually yellow pigmented bacteria that occur worldwide and cause disease on many plants. The host range spans over 124 monocotyledonous and 268 dicotyledonous plant species (Hayward, 1993, Leyns et al., 1984). Among the species of *Xanthomonas*, *X. campestris* is the most diverse and includes at least 125 different pathovars that are distinguished by causing different diseases of plants (Bradbury, 1984, Dye et al., 1980). Although the determination of the genus *Xanthomonas* and its species presents no great problem, the identification and characterization at the subgeneric level is still difficult (Van den Mooter and Swings, 1990, Vauterin et al., 1990a, 1991a, Young et al., 1992). The basis for differentiation of plant pathogenic bacteria at the subgeneric level is by the capability of the bacterial strain to cause characteristic disease or by reference to their host range (Dye et al., 1980, Vauterin et al., 1990a, Young et al., 1992). However, pathogenic reactions are variable. Strains may differ in aggressiveness, causing a broad range of symptoms when artificially inoculated into host plant species. The strains may also differ in the pathogenic reaction when artificially inoculated onto nonhost plants. Furthermore, these pathovars may not be readily differentiated by other phenotypic features (Van den Mooter and Swings, 1990). In addition, yellow-pigmented bacteria apparently belonging to the genus *Xanthomonas* and nonpathogenic to the plants from which they were isolated are usually associated with plant material (Angeles-Ramos et al., 1991, Gitaitis et al., 1987, Maas et al., 1985, Liao and Wells, 1987, Stall and Minsavage, 1990). These opportunistic bacteria can be identified as xanthomonads by the presence of xanthomonadins and by unique fatty acid profiles.

Attempts have been made to differentiate the pathovars and strains of *X. campestris* with several techniques. Traditional methods for the identification and characterization of plant pathogenic xanthomonads rely upon performing predetermined biochemical, serological, and pathological tests on pure cultures of the bacteria (Bradbury, 1984, Holt et al., 1994, Schaad, 1988). More recently, methods based on metabolic and protein profiles (Chase et al., 1992, Hildebrand et al., 1993, Van den Mooter and Swings, 1990, Vauterin et al., 1991ab) and fatty acid analysis (Chase et al., 1992, Yang et al., 1993) have been used for taxonomic and identification purposes. Polyclonal and monoclonal antisera produced against strains of *X. campestris* have been used for detection and identification, but they have provided variable results (Alvarez and Lou, 1982, Alvarez et al., 1991, Benedict et al., 1990, Jones et al., 1993b). Nucleic acid based techniques have become a premier approach for detection and identification of plant pathogenic bacteria (Bereswill et al., 1992, Manulis et al., 1991, Schaad et al., 1989, Seal et al., 1992), including members of the xanthomonads (Berthier et al., 1993, Garde and Bender, 1991, Gilbertson et al., 1989, Hartung, 1992, Hartung et al., 1993, Hildebrand et al., 1990, Lazo and Gabriel, 1987, Lazo et al., 1987, Leach et al., 1990,1992).

Genetic relationships among plant pathogenic xanthomonads have been established based on DNA-DNA homology, total genomic fingerprinting, and analysis of restriction fragment length polymorphism (RFLP) in their DNAs (Vauterin et al., 1990a). DNA-DNA hybridization studies have shown great variation in the relatedness of *X. campestris* pathovars (Hildebrand et al., 1990, Palleroni et al., 1993), and the strains of *X. campestris* were divided into 15 DNA homology groups (Vauterin et al., 1993). Comparisons by genomic fingerprinting and RFLP analysis have also shown significant genetic diversity among and within different pathovars of *X. campestris* (Egel et al., 1991; Lazo et al., 1987; Stall et al., 1994). Furthermore, physiologically and biochemically similar xanthomonads that are pathogenic on the same host plant may have a diverse genetic background (Egel et al., 1991; Hildebrand et al., 1990; Palleroni et al., 1993; Stall et al., 1994). On the other hand, the application of conventional genetic analysis for routine identification, as well as for taxonomic purposes, may be hampered by some limiting features. The determination of DNA-DNA homologies are limited by technical difficulties and by experimental error in obtaining the data (Smith, 1989). Further, rearrangements of the bacterial chromosome may result in changes in the phenotype and RFLP patterns without appreciably affecting overall sequence similarity (Egel et al., 1991; Young et al., 1992). Alternatively, analysis of specific DNA sequences of the bacterial genome should provide accurate information regarding the relationships of the strains and pathovars of *X. campestris* without the shortcomings mentioned above. The procedure may be a useful tool for the specific detection and identification of plant pathogenic bacteria.

The primary objective of this work is to investigate specific sequences of the bacterial genome of plant pathogenic xanthomonads for the specific identification of these plant pathogens. Oligonucleotide primers were designed based on the sequence of the *hrp* gene (hypersensitive reaction and pathogenicity) cluster of *X. campestris* pv vesicatoria (Bonas et al., 1991; Ulla Bonas, personal communication), and tested for the specific amplification of DNA fragments related to the *hrp* gene cluster from the bacterial genome of different plant pathogenic xanthomonads by polymerase chain reaction. DNA fragments related to the *hrp* genes were amplified from strains of *X.*

fragariae and of 28 pathovars of *X. campestris*. DNA sequence variation in the *hrp* related fragments was investigated by restriction endonuclease analysis, and the evolutionary relationship of these *hrp* gene sequences was assessed based on phylogenetic analysis of the restriction fragment data. The DNA amplification technique was also examined for specific detection and identification of plant pathogenic xanthomonads and to detect plant pathogens associated with plant samples.

CHAPTER 2
REVIEW OF LITERATURE

The genus *Xanthomonas* includes six species containing plant pathogenic bacteria (Bradbury, 1984 1986) that occur worldwide and cause economically important diseases on several plant crops The species *X campestris* is the largest and consists of at least 125 different pathovars (Bradbury, 1984, 1986) The basis for differentiation and identification of these plant pathogenic bacteria at the pathovar level is by means of the capability of the bacterial strain to cause characteristic disease or by their host range (Dye et al. 1980 Vauterin et al , 1990a, Young et al , 1992) The identification of the xanthomonads at the genus and species level does not present a significant problem, and can be achieved by using different techniques, such as biochemical and physiological tests, serology, protein profiling, and fatty acid and nucleic acid analysis (Bradbury 1984, Holt et al 1994 Schaad, 1988, Vauterin et al 1990ab). Despite a range of different techniques available the differentiation at infrasubspecific level is still difficult, and it is not uncommon that strains belonging to different pathovars may be genetically and phenotypically closely related.

Serology

Several attempts have been made to distinguish between pathovars of *X. campestris* based on serological reactions. Early work on the use of serology for differentiation of plant pathogenic xanthomonads by using agglutination and precipitation tests suggested that this technique would be specific for differentiation and identification of this group of bacteria (Link and Sharp, 1927 Williams and Glass, 1931) Elrod and Braun (1947) carried out an extensive study which included different species of *Xanthomonas* and several pathovars of *X campestris*, thus identifying five immunological groups which have a high degree of serological homogeneity within them However the serogroups did not show good or significant correlation with nomenspecies and pathotypes Subsequent work has confirmed the complex nature of the serological relationships among different taxonomic groups of the plant pathogenic xanthomonads

Polyclonal antibodies produced against whole cells of *X campestris* pv campestris cross-reacted with strains of *X campestris* pv armoraciae, *X campestris* pv phaseoli "fuscans" and *X. campestris* pv vesicatoria (Franken et al 1992). In immunofluorescence microscopy these polyclonal antibodies also cross-reacted with non xanthomonad bacteria. In a similar study polyclonal antibodies produced to whole cells of *X. campestris* pv pelargonii also reacted to strains of seven different pathovars of *X campestris* (Anderson and Nameth, 1990) Conflicting data have also emerged regarding the relationship between serotypes and pathotypes. O'Brien et al (1967) distinguished pepper strains from tomato strains of *X. campestris* pv vesicatoria by serological reaction, but no correlation between serotype and pathotype of *X. campestris* pv vesicatoria was found by Charudattan et al (1973) They found that tomato and pepper strains of the bacterium included both serotypes Further, no correlation was observed for hydrolysis of starch and resistance to streptomycin with serotypes. These contrasting results are not exclusive to *X campestris* pv vesicatoria, but have also been observed for other pathovars of *X. campestris* (Bach et al., 1978, Civerolo and Fan, 1982) and even to other plant pathogenic species of *Xanthomonas* (Obata and Tsuboi 1972, Mohanty and Addy 1977) Furthermore, the heterogeneous nature of antigenic determinants in immunogens from whole cell preparations is likely to play a major role in the low specificity of polyclonal antibodies. Consequently, cross reactions with different plant pathogenic xanthomonads pose a limitation in the identification of specific pathovars of *X. campestris* when these antibodies are used.

Attempts have been made to increase specificity of polyclonal antisera by different preparations of immunogens or by partially purified antigen preparations. Thaveechai and Schaad (1984) compared four different immunogen preparations of whole bacterial cells. However, none of the immunogens was specific enough in immunofluorescence to differentiate *X. campestris pv.* campestris from other pathovars of *X. campestris* (Thaveechai and Schaad, 1984). Partially purified antigens including enzymes, membrane proteins, and ribosomes have also been used as immunogens. However, polyclonal antisera produced against partially purified antigens have not provided enough specificity to distinguish among different pathovars of *X. campestris*. Thaveechai and Schaad (1984) typed 25 strains of *X. campestris pv.* campestris in four serotypes by using antisera produced against ribosomes. However, the antiserum cross-reacted with strains of *X. campestris pv.* malvacearum, *X. campestris pv.* translucens, and *X. campestris pv.* vesicatoria (Thaveechai and Schaad, 1984). Similar results were obtained with polyclonal antisera produced against membrane proteins of *X. campestris* pv. translucens (Azad and Schaad, 1988). Although strains of *X. campestris pv.* translucens from different gramineous hosts were grouped in two serotypes by using antisera to membrane proteins, *X. campestris* pv. begoniae was serologically indistinguishable from *X. campestris* pv. translucens in Ouchterlony and immunofluorescence tests (Azad and Schaad, 1988). Further, no correlation was observed between host of origin and serotype (Azad and Schaad, 1988).

Monoclonal antibodies (MABs) have been examined quite extensively for specific identification of plant pathogenic xanthomonads. MABs have been produced for various groups of these plant pathogens, including *X albilineans*, *X. oryzae* and several pathovars of *X. campestris* (Vauterin et al , 1993) Further, MABs have been developed for identification of xanthomonads at different taxonomic levels (Alvarez et al , 1985 Benedict et al , 1989, 1990). Alvarez et al (1985) obtained a MAB that identifies the genus *Xanthomonas* This MAB was specific for all 436 xanthomonads tested, but did not react with bacteria of 12 other genera. MABs were produced that were specific for *X oryzae* pv oryzae and for *X oryzae* pv oryzicola at pathovar level (Benedict et al 1989) These pathovar-specific antibodies reacted with all tested strains of each pathovar but neither of the MABs reacted with strains of other pathovars and species, or with strains of other bacterial genera. Also for MABs specific for epitopes on the lipopolysaccharide of *X. campestris* pv begoniae and *X. campestris* pv pelargonii were produced that were pathovar-specific and reacted only with their respective strains (Benedict et al 1990) These monoclonal antibodies did not react with strains of other xanthomonads or non xanthomonads tested

Contrary to the pathovar-specific MABs mentioned above, no pathovar specific MABs have been found that could be used to specifically detect all strains within certain pathovars of *X campestris* such as *X campestris* pv campestris (Alvarez et al. 1985, Franken et al., 1992) *X. campestris* pv citri (Alvarez et al , 1991) *X campestris* pv citrumelo (Alvarez et al. 1991 Gottwald et al., 1991, Permar and Gottwald, 1989), and *X campestris* pv dieffenbachiae (Lipp et al., 1992) Based on the reactions of six antibodies, 200 strains of *X campestris* pv campestris were grouped in six serogroups (Alvarez et al , 1991) However, this grouping did not imply closer genetic relationships among members of a group than with members of other groups or geographical distribution of the strains. In a similar study, six MABs were used to group 323 strains of *X. campestris* pv. dieffenbachiae isolated from different plants of the Araceae family (Lipp et al., 1992). The strains of *X. campestris* pv. dieffenbachiae were serologically heterogeneous and 12 major serogroups were identified. The serotype did not correspond strongly to the host of origin. Strains of *X. campestris* that cause diseases on citrus are also serologically diverse (Alvarez et al., 1990, 1991; Benedict et al., 1985; Gottwald et al., 1991; Permar and Gottwald, 1989). An MAB developed to *X. campestris* pv. citri of the group A of citrus canker did not react with strains of B, C, and D forms of citrus canker (Alvarez et al., 1991). Although this antibody also did not react with 130 other *Xanthomonas* pathovars and species, it reacted with some strains of *X. campestris* pv. citrumelo, the citrus bacterial spot agent, and with a strain of *X. campestris* pv. manihotis. However, in serological studies with monoclonal and polyclonal antibodies a close antigenic relationship existed between the strains of the groups B, C, and D forms of citrus canker (Alvarez et al., 1991; Civerolo and Fan, 1982). The citrus bacterial spot pathogen, *X. campestris* pv. citrumelo, is also serologically heterogeneous, and no single MAB reacted with all strains of this pathogen (Alvarez et al., 1991; Gottwald et al., 1991). Although *X. campestris* pv. citrumelo is serologically distinct from strains of *X. campestris* pv. citri, some strains of the citrus bacterial spot pathogen share a common epitope with members of other pathovars of *X. campestris* (Alvarez et al., 1991; Gottwald et al., 1991). The diversity of *X. campestris* pv. citrumelo is also reflected genetically and in differential host reactions (Alvarez et al., 1991; Egel et al., 1991; Gottwald et al., 1991; Graham and Gottwald, 1990).

An interesting point emerging from these studies on serological relationships among different pathovars of *X. campestris* by using MABs is the inverse correlation between the serological heterogeneity and the host range of the xanthomonads. Pathovar specific MABs have been developed that react specifically with all strains of a pathovar of *X. campestris* that usually cause disease in a single host, as for example, *X. campestris* pv. begoniae and *X. campestris* pv. pelargonii. On the contrary, no pathovar specific MABs were produced that react with all strains of a pathovar that have a large host range, such as *X. campestris* pv. campestris, *X. campestris* pv. citri, and *X. campestris* pv. dieffenbachiae.

Fatty Acids Composition

The analysis of fatty acids by gas chromatography has become a common technique in bacterial classification and identification. Comprehensive studies have been carried out on the relationship of fatty acid profiles within several groups of plant pathogenic xanthomonads (Chase et al., 1992, Graham et al., 1990, Hodge et al., 1992, Vauterin et al., 1991b, 1992, Yang et al., 1993). At least 65 different fatty acids were found within the members of the genus *Xanthomonas*. Three fatty acids, 11:0 iso, 11:0 iso 3OH, and 13:0 iso OH, are characteristic for all xanthomonads and they were useful for differentiating *Xanthomonas* from other plant pathogenic bacteria (Vauterin et al., 1992, Yang et al., 1993). Furthermore, the species *X. albilineans*, *X. fragariae*, and *X. populi* were homogeneous based on fatty acid composition, and they could be clearly differentiated from each other (Hodge et al., 1992, Vauterin et al., 1992, Yang et al., 1993). Strains of *X. oryzae* have unique fatty acid composition within the genus *Xanthomonas* and they form two major groups which correspond to the pathovars *X. oryzae* pv. oryzae and *X. oryzae* pv. oryzicola (Vauterin et al., 1992, Yang et al., 1993).

As observed on other characteristics, *X. campestris* also comprises a heterogeneous group in relation to the fatty acid composition (Chase et al., 1992, Hodge et al., 1992, Vauterin et al., 1991b 1992 Yang et al., 1993). Extensive studies carried out to study the fatty acid composition of strains of the genus *Xanthomonas* included bacteria from more than 100 pathovars of *X. campestris* (Hodge et al., 1992, Yang et al., 1993). The members of the *X. campestris* have been divided in 24 different groups on the basis of fatty acid composition (Yang et al., 1992). Some pathovars of *X. campestris*, such as *X. campestris* pv. maculifoliigardeniae, *X. campestris* pv. malvacearum, and *X. campestris* pv. pelargonii, were homogeneous and had a distinct fatty acid profiles (Hodge et al., 1992). In some cases, pathovars pathogenic for members of the same family of plants could be grouped together based on fatty acid composition. This is the case for the pathovars *X. campestris* pv. cerealis, *X. campestris* pv. hordei, *X. campestris* pv. secalis, *X. campestris* pv. translucens, and *X. campestris* pv. undulosa which cause diseases on cereal crops (Vauterin et al., 1992; Yang et al., 1993), and also for the pathovars from crucifers *X. campestris* pv. aberrans, *X. campestris* pv. armoraciae *X. campestris* pv. barbareae, *X. campestris* pv. campestris, *X. campestris* pv. incanae, and *X. campestris* pv. raphani (Yang et al., 1993). Many pathovars from leguminous plants such as *X. campestris* pv. alfalfae, *X. campestris* pv. glycines, *X. campestris* pv. phaseoli *X. campestris* pv. phaseoli "fuscans", *X. campestris* pv. rhynchosiae *X. campestris* pv. sesbaniae and *X. campestris* pv. vignicola, also clustered in the same group based on fatty acid analysis (Yang et al., 1993). This grouping delineated on the basis of fatty acid composition agreed with the relationships established based on DNA-DNA hybridization and protein profile (Hildebrand et al., 1990; Vauterin et al., 1991a, 1992, Yang et al., 1993).

In contrast to some pathovars, the strains of *X. campestris* causing diseases of citrus are composed of a heterogeneous group of strains. Besides the heterogeneity in the fatty acid profile, the grouping obtained by fatty acid analysis does not agree entirely with the grouping obtained by using other methods, such as by genetic analysis and phenotypic features. Strains of *X. campestris* pv. citri that cause the citrus canker disease can be clearly differentiated from strains of *X. campestris* pv. citrumelo, the citrus bacterial spot pathogen (Vauterin et al., 1991b, Yang et al., 1993). However, there are some discrepancies between the delineation of the citrus canker groups by fatty acid profile compared to genetic analysis and protein profile (Vauterin et al., 1991b). Based on fatty acid analysis, the strains of citrus canker B were indistinguishable from strains of the citrus canker A, but they were distinct from the strains of groups C and D of citrus canker (Vauterin et al., 1991b). However, the citrus canker A strains are considered a homogeneous and distinct group from the strains of citrus canker B, C, and D based on DNA-DNA hybridization, RFLP analysis, and protein profiles (Egel et al., 1991, Gabriel et al., 1989; Hartung and Civerolo, 1987, Vauterin et al., 1991b). Contrasting results on fatty acid analysis were also obtained for the citrus bacterial pathogen *X. campestris* pv. citrumelo. Vauterin et al. (1991b) found that strains within *X. campestris* pv. citrumelo constituted a distinct and homogeneous group based on fatty acid composition. On the other hand, Graham et al. (1990) observed considerable diversity among the *X. campestris* pv. citrumelo strains, and some of these strains were closely related to non citrus strains of *X. campestris* on the basis of fatty acid composition.

Protein Profiles

El-Sharkawy and Huisingh (1971ab) were the first to demonstrate the usefulness of electrophoresis of proteins for differentiation and identification of *Xanthomonas*. The patterns of native negatively charged proteins extracted from whole bacterial cells varied considerably and indicated the occurrence of taxon-specific protein profiles that could be used for differentiation and identification of plant pathogenic xanthomonads. The presence of distinct and specific patterns of esterases at the pathovar level was particularly valuable in differentiating and identifying the different groups of plant pathogenic *Xanthomonas* spp (El-Sharkawy and Huisingh, 1971a) However, a more reliable and reproducible techniques were required to resolve the protein profile at subspecific level In this respect, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) of total cellular proteins has become a key technique for the separation and characterization of a wide variety of proteins (Laemmli 1970 Weber and Osborn, 1969) Furthermore, this technique has been useful for differentiating the plant pathogenic xanthomonads on the basis of whole-cell protein (Bouzar et al , 1994 Kersters and De Ley 1975, Van den Mooter et al., 1987ab, Vauterin et al., 1990a, 1991ab, 1992) Variation in the electrophoretic patterns of specific proteins of the bacterial cell such as total cell envelope and total membrane proteins, has also been investigated by using SDS PAGE for differentiation of some pathovars of *X campestris* (Dos Santos and Dianese, 1985, Minsavage and Schaad, 1983, Qhobela and Claflin, 1992, Qhobela et al , 1991)

Vauterin et al (1991a) analyzed whole-cell proteins of an extensive collection of plant pathogenic bacteria of the genus *Xanthomonas*, comprising all species and 27 pathovars of *X campestris*, by using SDS-PAGE analysis. On the basis of SDS-PAGE protein profiles the plant pathogenic species *X albilineans*, *X axonopodis*, *X. fragariae*, and *X. oryzae* could be distinguished from each other and also from *X. campestris* pathovars whereas *X populi* was rather similar to *X. campestris* Several distinct protein profiles were observed within *X campestris*, and some of the groups established corresponded to described pathovars of this species. Certain pathovars constituted distinct and homogeneous groups based on protein profiles, such as *X. campestris* pv. campestris, *X. campestris* pv. graminis, *X. campestris* pv. hyacinthi, *X. campestris* pv. pelargonii, *X. campestris* pv. pruni and *X. campestris* pv. theicola. On the other hand, the pathovars *X. campestris* pv. alfalfae, *X. campestris* pv. cajani, *X. campestris* pv. glycines, *X. campestris* pv. phaseoli, *X. campestris* pv. phaseoli "fuscans" and *X. campestris* pv. vignicola which cause diseases on leguminous plants could not be differentiated from one another. The relatedness of this group of pathogens of leguminous plants has also been confirmed by DNA-DNA hybridization (Hildebrand et al., 1990, Vauterin et al., 1990ab) and by fatty acid composition (Yang et al., 1993). Moreover, some pathovars, including *X. campestris* pv. alfalfae, *X. campestris* pv. citri, *X. campestris* pv. dieffenbachiae, *X. campestris* pv. poinsettiicola, *X. campestris* pv. vesicatoria, *X. campestris* pv. vignicola, and *X. campestris* pv. vitians, were heterogeneous on the basis of protein profiles, consisting of two or more distinct protein groups (Vauterin et al., 1991b). The diversity on the basis of protein profile for some of these pathovars, i.e. *X. campestris* pv. citri, *X. campestris* pv. dieffenbachiae, and *X. campestris* pv. vesicatoria, correlates with the heterogeneity based on genetic analysis and fatty acid composition (Chase et al., 1992, Egel et al., 1991, Graham et al., 1990, Stall et al., 1994, Vauterin et al., 1991ab).

The complexity of the grouping of strains of *X. campestris* that cause diseases on citrus on the basis of whole-cell protein profiles (Vauterin et al., 1991ab) is in agreement with the diversity determined by genetic analysis (Egel et al., 1991, Gabriel et al., 1989, Hartung and Civerolo, 1987) and fatty acid composition (Graham et al., 1990, Vauterin et al., 1991b). Based on protein profile, the strains of the citrus canker A of *X. campestris* pv. citri form a well defined and homogeneous group (Vauterin et al., 1991b). The uniformity of this group was also revealed by DNA-DNA hybridization, DNA fingerprinting, RFLP analyses, and fatty acid composition (Egel et al., 1991, Gabriel et al., 1987, Graham et al., 1990, Hartung and Civerolo, 1987, Vauterin et al., 1991b). The strains of the citrus canker B and D groups of *X campestris* pv. citri constituted one group, and strains of citrus canker C formed another distinct group on the basis of protein profile (Vauterin et al., 1991b). However, the groupings based on DNA-DNA hybridization, fatty acid composition, and RFLP analysis do not entirely agree with the groups determined by protein analysis (Vauterin et al., 1991b). Although distinct from the citrus canker A strains of *X. campestris* pv. citri, strains of *X. campestris* pv. citrumelo also showed a quite uniform protein patterns on the SDS-PAGE (Vauterin et al., 1991b). This contrasts with the diversity determined for the strains of this pathovar based on fatty acid composition (Graham et al., 1990), RFLP analyses (Gabriel et al., 1989, Hartung and Civerolo, 1987), and DNA-DNA hybridization (Egel et al., 1991).

SDS-PAGE of whole-cell proteins was also used in comparative studies of the xanthomonads that cause diseases on cassava, *X. campestris* pv. manihotis and *X. campestris* pv. cassavae (Van den Mooter et al., 1987a). Strains of *X. campestris* pv. manihotis constituted a distinct and homogeneous group on the basis of protein profiles, whereas the strains of *X. campestris* pv. cassavae formed two groups. These results also agree with the groupings obtained by numerical analyses of phenotypic features (Van den Mooter et al., 1987a), DNA-DNA hybridization (Van den Mooter et al., 1987a), and electrophoresis of total membrane proteins (Dos Santos and Dianese 1985).

X. campestris pv. vesicatoria, the cause of the bacterial spot disease of tomato and pepper, comprises heterogeneous groups in relation to protein profiles (Bouzar et al., 1994, Vauterin et al., 1991a). This diversity has also been confirmed by genetic analyses and phenotypic characteristics (Stall et al., 1994; Vauterin et al., 1990a). Two major distinct groups have been delineated by analysis of whole-cell protein profiles (Bouzar et al., 1994; Vauterin et al., 1991a), utilization of carbon sources (Bouzar et al., 1992), fatty acid composition (Stall et al., 1994; Vauterin et al., 1990b) and DNA-DNA hybridization (Stall et al., 1994). Furthermore, these two groups can be easily distinguished by the presence of unique protein bands on the basis of silver staining of SDS-lysed cell proteins (Bouzar et al., 1994).

A comparative study was carried out of the *Xanthomonas* species and *X. campestris* pathovars that cause diseases on cereals and grasses by using SDS-PAGE of whole-cell proteins (Vauterin et al., 1992). The pathovars of the "translucens" group which includes *X. campestris* pv. cerealis, *X. campestris* pv. hordei, *X. campestris* pv. secalis, *X. campestris* pv. translucens, and *X. campestris* pv. undulosa, could not be distinguished from one another. The pathogens of grasses which formed another homogeneous group were *X. campestris* pv. arrhenatheri, *X. campestris* pv. phlei, and *X. campestris* pv. poae, and had protein profiles very similar to those of the "translucens" group. DNA-DNA hybridization data, phenotypic features, and fatty acid composition also support the close relationship between the two groups (Van den Mooter et al., 1987b; Vauterin et al., 1992). On the other hand, *X. axonopodis* and *X. campestris* pv. vasculorum were heterogeneous, with two distinct groups being found in each taxon. The type A group of *X. axonopodis* was very closely related to one group of *X. campestris* pv. vasculorum, whereas the type B group of *X. axonopodis* had atypical protein and fatty acid profiles which do not resemble xanthomonads. *X. campestris* pv. vasculorum type B group was indistinguishable from *X. campestris* pv. holcicola based on protein profile, as well as on fatty acid composition and DNA-DNA hybridization (Vauterin et al., 1992).

Phenotypic Characteristics

The differentiation and identification of *Xanthomonas* spp. have largely depended on phenotypic characteristics (Bradbury, 1984, Holt et al., 1994, Schaad, 1988). Although members of the xanthomonads can be distinguished from the bacteria of other genera on the basis of phenotypic features (Bradbury, 1984, Holt et al., 1994, Schaad, 1988), it has been almost impossible to differentiate within the *Xanthomonas* by biochemical and physiological features alone without knowing their plant hosts (Bradbury, 1984, Dye, 1962, Holt et al., 1994, Schaad, 1988, Van den Mooter and Swings, 1990). In fact, this was the major reason for the inclusion of the majority of the former species of *Xanthomonas* into a single species, *X. campestris* (Bradbury, 1984, Dye et al., 1980).

Extensive studies have been carried out to compare the phenotypic characteristics of strains representing the different species of the genus *Xanthomonas*, pathovars of *X. campestris*, and other bacteria genetically related to the xanthomonads (Dye, 1962, Hildebrand et al., 1993, Van den Mooter and Swings, 1990). Van den Mooter and Swings (1990) examined 295 morphological, biochemical, and physiological features of 266 strains of *Xanthomonas* spp. and related bacteria. The plant pathogenic xanthomonads were placed in seven different groups by cluster analysis. The groups were delineated by strains of the taxa *X. albilineans*, *X. axonopodis*, *X. campestris*, *X. campestris* pv. graminis, *X. fragariae*, *X. oryzae*, and *X. populi*, which largely correlate with the existing species of the genus *Xanthomonas*. Strains of *X. campestris* pv. graminis did not cluster with other pathovars of *X. campestris*, and they were considered a homogeneous and different group on the basis of phenotypic characteristics. However, other studies of phenotypic features (Van den

Mooter et al., 1987b), DNA-DNA hybridization (Kersters et al., 1989, Vauterin et al., 1992), SDS-PAGE (Van den Mooter et al., 1987b, Vauterin et al., 1992), and fatty acid profiles (Vauterin et al., 1992, Yang et al. 1993) have failed to consistently differentiate strains of *X. campestris* pv. graminis from other pathovars of *X. campestris* which cause diseases on cereals and grasses, such as *X. campestris* pv. arrhenatheri, *X. campestris* pv. cerealis, *X. campestris* pv. hordei, *X. campestris* pv. phlei, *X. campestris* pv. phleipratensis, *X. campestris* pv. poae, *X. campestris* pv. secalis, *X. campestris* pv. translucens, and *X. campestris* pv. undulosa. Furthermore, the misnamed species *Pseudomonas gardneri*, *P. vitiswoodrowii*, and *P. mangiferaeindicae* were indistinguishable from *X. campestris* strains on the basis of phenotypic features (Dye, 1966, Hildebrand et al., 1993, Van den Mooter and Swings, 1990). The latter also agrees with studies of DNA-rRNA hybridization (De Vos et al., 1985), DNA-DNA hybridization (Hildebrand et al., 1990, Palleroni et al., 1993), and fatty acid composition (Yang et al., 1993).

X. campestris contains by far the largest group of strains within the genus *Xanthomonas*, consisting of at least 125 different pathovars (Bradbury, 1984). Despite certain variations in phenotypic characteristics (Hildebrand et al., 1993), strains of *X. campestris* form a rather indistinguishable group (Burkholder and Starr, 1948, Dye, 1962, Van den Mooter and Swings, 1990). In a limited number of cases, however, distinctive phenotypic traits have been linked to the grouping based on pathogenicity to a given host species. Two of these cases are the *X. campestris* pv. manihotis and *X. campestris* pv. cassavae, which cause diseases on cassava. All strains of each pathovar formed a single group based on phenotypic features (Van den Mooter and Swings, 1990, Van den Mooter et al., 1987a). Correlation between phenotypic features and pathogenic specialization was also established for the xanthomonads that cause wilt diseases on grasses. The analysis of 257 phenotypic characteristics of strains from grasses revealed three distinct groups corresponding to (1) *X. campestris* pv. graminis, and *X. campestris* pv. phleipratensis, (2) *X. campestris* pv. arrhenatheri, and (3) *X. campestris* pv. poae (Van den Mooter et al., 1987b). These groups are also in agreement with the ones established on the basis of pathogenicity features. However, most of the strains of pathovars of *X. campestris* have heterogeneous phenotypic characteristics and only have the pathogenicity to the same host plant as the common feature (Burkholder and Starr, 1948, Dye, 1962, Van den Mooter and Swings, 1990). This can be illustrated by the case of *X. campestris* pv. dieffenbachiae. Chase et al. (1992) analyzed the phenotypic features of 149 strains of *X. campestris* pv. dieffenbachiae obtained from aroid plants and found large variation in their reactions. Minimum pH for growth in oatroot broth was the only useful feature to differentiate typical strains obtained from *Syngonium* spp. from all other aroid strains. Although some trends were observed, physiological tests, such as hydrolysis of both pectin and starch, and growth on different media, failed to differentiate the strains to their host of origin. The strains of *X. campestris* pv. dieffenbachiae from different hosts overlapped in their reactions for these tests, which made it impossible to relate the phenotypic features to the host of origin.

Commercially available systems for identification of bacteria on the basis of phenotypic characteristics have also been examined for differentiation of plant pathogenic xanthomonads (Chase et al., 1992, Jones et al., 1993a, Vauterin et al., 1990b, Verniere et al., 1993). The Biolog GN MicroPlate System, which is based on differential utilization of 95 carbon sources with consequent changes in redox potential of the bacterial suspension (Bochner, 1989), was evaluated for accuracy in identifying some plant pathogenic bacteria, including strains of *Xanthomonas* (Jones et al., 1993a)

The identification of strains of *Xanthomonas* to species and to pathovar was correct in 97% and 6% of the cases, respectively, when the original data base was used However, the accuracy of the Biolog system for identification of pathovars of *X campestris* was substantially improved when the database was amended by the addition of data obtained for different strains of *X. campestris* Nevertheless, the Biolog results confirmed the close relatedness between the various pathovars determined in previous work, and substantiated the difficulty of separation of pathovars on the basis of phenotypic features alone

The metabolic variation of strains of *X campestris* that cause diseases on citrus was investigated by using the Biolog system (Hartung and Civerolo, 1991, Verniere et al , 1993) The Biolog metabolic profile was useful for differentiating strains of the citrus canker pathogen *X campestris* pv citri from strains of the citrus bacterial spot agent *X campestris* pv citrumelo Furthermore the different citrus canker groups of *X campestris* pv citri could be differentiated based on the utilization of three carbon sources (Verniere et al 1993) Although the correct identification of the *X campestris* pv citri strains was very poor by using the original data base of the system, the identification was significantly improved when the database was supplemented with additional data (Verniere et al , 1993) The citrus bacterial spot pathogen *X. campestris* pv citrumelo was more variable in the metabolic profile than *X campestris* pv citri (Hartung and Civerolo, 1991) All the profiles examined placed the strains within the species *X campestris* However, some strains were identified as *X campestris* pv dieffenbachiae (Hartung and Civerolo, 1991) The relatedness of strains of *X. campestris* pv citrumelo to other pathovars of *X campestris* has also been determined on the basis of DNA-DNA hybridization (Egel et al , 1991) RFLP analysis (Gabriel et al , 1988 1989), and fatty acid composition (Graham et al , 1990)

The enzymatic API ZYM galleries (API Systems S A., La-Balme-les-Grottes Montalieu-Vercieu, France), has also been tested for differentiation and identification of plant pathogenic xanthomonads (Vauterin et al , 1990b) The enzymatic profiles of *X. campestris* pv begoniae and *X. campestris* pv pelargonii were investigated with the API ZYM system. The profiles were highly uniform within each pathovar and they could be differentiated by three enzymatic activities, chymotrypsin, α-D-galactosidase, and α-D-glucosidase However, the API ZYM system did not seem to be discriminative at the pathovar level when larger numbers of pathovars were included in the study (Vauterin et al 1993).

Nucleic Acids Analysis

A number of techniques have been used for characterization of nucleic acids of the xanthomonads DNA-rRNA hybridization has been of major importance in differentiation of the xanthomonads at the intrageneric level (De Vos and De Ley 1983, Palleroni et al 1973), whereas DNA DNA hybridization has become the basis for determining genetic relationships among bacterial strains and for establishing classification schemes at the subgeneric level (Holt et al., 1994, Krieg and Holt, 1984, Wayne et al 1987) Furthermore DNA-DNA reassociation is considered to be the reference standard to determine the phylogeny and consequently the taxonomy of bacteria (Wayne et al 1987) RFLP analysis of genomic DNA has been useful to distinguish several groups of xanthomonads, to study genetic diversity and to establish phylogenetic relationships among different pathovars of *X. campestris* (Gabriel et al., 1988, 1989 Gottwald et al., 1991, Graham et al , 1990, Hartung and Civerolo, 1989, 1991, Lazo and Gabriel, 1987 Lazo et al 1987 Leach et al 1990, 1992 Qhobela and Claflin, 1992 Qhobela et al. 1991 Verdier et al., 1993).

Nucleic acid based techniques have also been applied to the identification and detection of the plant pathogenic xanthomonads. Genomic and plasmid DNA fingerprinting has been used in several instances for the differentiation of a number of groups of xanthomonads (Berthier et al., 1993, Cooksey and Graham, 1989, Hartung and Civerolo, 1987, Pruvost et al., 1992). In the same way, genomic and plasmid DNA probes were also developed and examined for the purpose of identification and detection of plant pathogenic xanthomonads (Garde and Bender, 1991, Gilbertson et al., 1989, Hartung, 1992, Lazo and Gabriel, 1987, Leach et al., 1990, 1992).

The genomic relationship among plant pathogenic xanthomonads determined by DNA-DNA hybridization has revealed a group of bacteria with diverse genetic background, with DNA homologies between pathovars of *X. campestris* ranging from values as low as 15% to 39% (Vauterin et al., 1990b). These results strongly reinforce the contention that strains of *X. campestris* do not constitute a single species. Furthermore, the genetic relationship established on the basis of DNA-DNA homology does not always correlate with the pathogenicity of the bacteria toward a given range of host plants. The genetic relationship of strains of plant pathogenic xanthomonads representing 44 *X. campestris* pathovars and *Xanthomonas* spp. was examined by S1 DNA-DNA hybridization (Hildebrand et al., 1990, Palleroni et al., 1993). Several groups of xanthomonads were defined on the basis of DNA-DNA homology. The largest group consisted of strains belonging to 25 pathovars of *X. campestris* (Hildebrand et al., 1990, Palleroni et al., 1993). This group corresponds to DNA homology group VIII established by Vauterin et al. (1993), which also includes 22 additional pathovars of *X. campestris* and *X. axonopodis*. Another cluster comprises a group of *X. campestris* pv. carotae, *X. campestris* pv. gardneri, *X. campestris* pv. pelargonii, and *X. campestris* pv. taraxaci (Hildebrand et al., 1990, Palleroni et al.,

1993) Two minor clusters were also established. One group comprises the pathovars *X campestris* pv campestris, *X campestris* pv plantaginis, and *X. campestris* pv raphani, and the other group comprises *X campestris* pv celebensis and *X. campestris* pv juglandis (Palleroni et al , 1993).

Although the genetic relatedness among plant pathogenic xanthomonads which cause disease in different plants does not support a correlation between genomic groupings and pathogenicity features there are some exceptions. The pathovars of *X. campestris* which cause diseases on closely related leguminous plants, *X campestris* pv glycines, *X. campestris* pv lespedezae *X campestris* pv phaseoli, *X campestris* pv phaseoli "fuscans" and *X. campestris* pv vignicola, cluster in DNA homology group VIII of Vauterin et al (1993), *X campestris* pv pisi was the only leguminous pathogen not included in this group (Hildebrand et al., 1990, Palleroni et al., 1993 Vauterin et al , 1993) However, the leguminous pathogens are also genetically highly related to strains of several pathovars of *X. campestris* that cause diseases on different plants (Hildebrand et al , 1990, Palleroni et al 1993, Vauterin et al., 1993) The strains of *X campestris* pv arrhenatheri *X campestris* pv cerealis, *X campestris* pv graminis, *X. campestris* pv hordei, *X campestris* pv phlei, *X campestris* pv phleipratensis, *X campestris* pv poae *X. campestris* pv secalis, and *X campestris* pv translucens, that cause diseases on gramineous hosts cluster together in the DNA homology group IX of Vauterin et al (1993) This grouping also correlates strongly with groupings based on SDS-PAGE and fatty acid analysis (Vauterin et al 1992). The DNA homology group XII contains the six pathovars from crucifers, *X campestris* pv aberrans, *X campestris* pv armoraciae *X campestris* pv barbareae, *X. campestris* pv campestris, *X. campestris* pv incanae, and *X campestris* pv raphani (Vauterin et al , 1993)

The most striking feature of the plant pathogenic xanthomonads is the genetic diversity among strains of the same pathovar or different pathovars of *X. campestris* that cause diseases on highly related hosts. The diversity strongly illustrates the divergence between pathogenicity characteristics and genetic backgrounds. The pathovars *X. campestris* pv. gardneri, *X. campestris* pv. physalidicola, and *X. campestris* pv. vesicatoria, which cause diseases on solanaceous plants, are genetically diverse on the basis of DNA-DNA homology (Hildebrand et al., 1990; Palleroni et al. 1993; Stall et al. 1994; Vauterin et al. 1993). *X. campestris* pv. vesicatoria is composed of the two genetically unrelated groups A and B (Stall et al., 1994). *X. campestris* pv. vesicatoria group A belongs to homology group VIII of Vauterin et al. (1993), as well as *X. campestris* pv. physalidicola. Further, *X. campestris* pv. vesicatoria group A and *X. campestris* pv. physalidicola are also genetically closely related to several other pathovars of *X. campestris* that cause diseases on different plants (Hildebrand et al., 1990; Palleroni et al. 1993; Vauterin et al. 1993). Strains of *X. campestris* pv. vesicatoria group A are only about 33% related to strains of *X. campestris* pv. vesicatoria group B, on the basis of DNA-DNA homology (Stall et al., 1994). Strains of *X. campestris* pv. vesicatoria group B for instance is distinct from other xanthomonads and they are the only members of the DNA homology group XVI (Vauterin et al., 1993). The grouping of *X. campestris* pv. vesicatoria are also well supported by fatty acid analysis, serology and phenotypic features (Stall et al., 1994). *X. campestris* pv. gardneri is also not related genetically to the other solanaceous pathogens. Instead, *X. campestris* pv. gardneri constitutes a group which contains the pathovars *X. campestris* pv. carotae, *X. campestris* pv. pelargonii, and *X. campestris* pv. taraxaci (Hildebrand et al., 1990; Palleroni et al., 1993).

The xanthomonads that cause diseases on citrus also comprise a genetically diverse group of bacteria. Strains of the citrus pathogens *X. campestris* pv. citri and *X. campestris* pv. citrumelo which belong to the DNA homology group VIII of Vauterin et al. (1993) are only about 60% similar to one another based on DNA-DNA homology (Egel et al., 1991, Vauterin et al., 1991b). *X. campestris* pv. citri canker A strains are, however, highly related to other pathovars of *X. campestris*. The canker A strains of this bacterium are related at over 90% by DNA-DNA homology to *X. campestris* pv. glycines and *X. campestris* pv. malvacearum (Egel et al., 1991, Vauterin et al., 1993). On the other hand, strains of the citrus bacterial pathogen, *X. campestris* pv. citrumelo, are closely related to strains of other pathovars of *X. campestris* which includes *X. campestris* pv. alfalfae and *X. campestris* pv. fici (Egel et al., 1991, Vauterin et al., 1993). A strong relationship exists between the citrus pathogens based on corroborating data obtained from DNA-DNA homology studies and analyses of fatty acid composition (Graham et al., 1990, Vauterin et al., 1991b), SDS-PAGE of proteins (Vauterin et al., 1991b), and RFLP of genomic DNA (Gabriel et al., 1988, 1989, Graham et al., 1990, Vauterin et al., 1991b).

Different techniques of genomic fingerprinting have been used to explore the genetic diversity of the plant pathogenic xanthomonads for identification and comparison purposes. Genomic fingerprintings produced by using frequent-cutting endonucleases were examined for establishing the genetic relationships of the xanthomonads that cause diseases on citrus (Hartung and Civerolo, 1987, Pruvost et al., 1992). The citrus canker A and B groups of *X. campestris* pv. citri each produced very conserved genomic fingerprintings which distinguished strains of both groups (Hartung and Civerolo, 1987). Although strains of the citrus bacterial spot pathogen *X. campestris* pv. citrumelo could be distinguished from both canker groups of *X.*

campestris pv citri a wide diversity of genomic fingerprints (Hartung and Civerolo 1987) was found. The genetic diversity of *X campestris* pv citrumelo was also determined by analysis of restriction patterns derived from pulsed-field gel electrophoresis of genomic DNA fragments generated by rare-cutting endonucleases (Egel et al , 1991) In contrast to the diversity of the strains of *X campestris* pv citrumelo, strains of *X campestris* pv citri canker A and B produced characteristic restriction patterns by pulsed field gel electrophoresis (Egel et al., 1991)

Attempts have also been made to differentiate *X campestris* pathovars by RFLP analysis of plasmid and genomic DNAs based on hybridization with different DNA probes. In most of the cases, random cloned fragments of the bacterial genome were used as DNA probes in RFLP analysis of the xanthomonads Specific regions of the genome have been selected for DNA probes such as rRNA sequences (Berthier et al., 1993, DeParasis and Roth, 1990) repetitive DNA sequences (Leach et al , 1990 1992) copper resistance genes (Garde and Bender 1991) and plasmid DNA sequences (Hartung, 1992, Gilbertson et al , 1989 Lazo and Gabriel 1987) Lazo et al (1987) used a cloned DNA fragment derived from *X campestris* pv citrumelo for RFLP analysis to differentiate pathovars of *X campestris* The RFLP analysis involving strains representing 26 pathovars of *X campestris* revealed profiles highly conserved and characteristic for each pathovar tested. By using more than one DNA probe, or by digesting the genomic DNA with different restriction endonucleases, it was possible to differentiate all the pathovars of *X campestris* included in the study (Lazo et al., 1987). An rRNA probe was also used to distinguish pathovars of *X campestris* (Berthier et al 1993) Further the RFLP patterns established for the pathovars *X campestris* pv begoniae *X campestris* pv dieffenbachiae, *X campestris* pv malvacearum, and *X* *campestris* pv. manihotis correlated with pathogenicity on the host plant (Berthier et al., 1993).

Extensive RFLP analyses were conducted in some pathovars of *X. campestris*, such as *X. campestris* pv. citri and *X. campestris* pv. citrumelo (Gabriel et al., 1988, 1989, Gottwald et al., 1991, Graham et al., 1990, Hartung, 1992, Hartung and Civerolo, 1989, 1991, Lazo and Gabriel, 1987, Lazo et al., 1987), *X. campestris* pv. manihotis (Verdier et al., 1993), *X. campestris* pv. vasculorum (Qhobela and Claflin, 1992), *X. campestris* pv. pennamericanum (Qhobela and Claflin, 1988), and *X. oryzae* pv. oryzae (Leach et al., 1990, 1992). The most comprehensive RFLP analyses were carried out to determine the genetic diversity of the xanthomonads causing diseases of citrus (Gabriel et al., 1988, 1989, Gottwald et al., 1991, Graham et al., 1990, Hartung, 1992, Hartung and Civerolo, 1989, 1991, Lazo and Gabriel, 1987, Lazo et al., 1987). The RFLP analyses characterized the strains of the citrus bacterial spot pathogen, *X. campestris* pv. citrumelo, as distinct from all forms of the citrus canker pathogen (Gabriel et al., 1988, 1989, Hartung and Civerolo, 1989). In contrast to the characteristic restriction patterns of the *X. campestris* pv. citri canker groups A and B, *X. campestris* pv. citrumelo gave a wide range of variation in the RFLP analyses (Gabriel et al., 1988, 1989, Graham et al., 1990, Hartung and Civerolo, 1989, 1991). The RFLP analyses further supported that strains of the citrus bacterial spot pathogen are not genetically closely related to any recognized group of *X. campestris* pv. citri (Hartung and Civerolo, 1987, 1989, Gabriel et al., 1988, 1989). Instead, the analysis has revealed a significant genetic relationship between strains of *X. campestris* pv. citrumelo and strains of non-citrus pathogens, such as *X. campestris* pv. alfalfae and *X. campestris* pv. fici (Gabriel et al., 1988, 1989, Graham et al., 1990). The relatedness of strains of *X. campestris* pv. citrumelo to strains of the other pathovars of *X. campestris* was further supported on the basis of DNA homology and phenotypic characteristics (Egel et al., 1991, Graham et al., 1990).

Nucleic acid based techniques have also been examined for the specific detection of plant pathogenic xanthomonads (Garde and Bender, 1991, Gilbertson et al., 1989, Hartung, 1992, Hartung et al., 1993). Garde and Bender (1991) developed DNA probes for detection of copper resistance in *X. campestris* pv. vesicatoria. Although the DNA probes seem specific to the genes conferring resistance to copper in *X. campestris* pv. vesicatoria and apparently did not hybridize to DNA of copper resistant *P. syringae* pv. tomato (Garde and Bender 1991), the *cop* operon of *P. syringae* pv. tomato not only hybridized to copper sensitive bacteria (Cooksey et al., 1990), but also to strains of *X. campestris* pv. vesicatoria (Voloudakis et al., 1993). In another study Gilbertson et al. (1989) used DNA probes obtained from a cryptic plasmid for specific detection of the bean pathogens *X. campestris* pv. phaseoli and *X. campestris* pv. phaseoli "fuscans". Although the DNA probes did not hybridize to nonpathogenic xanthomonads isolated from bean plants, they did hybridize to total genomic DNA of bacterial strains of other pathovars of *X. campestris* (Gilbertson et al. 1989). However, strains of these other pathovars of *X. campestris* were thought unlikely to be associated with bean plants. Plasmid based DNA probes have also been used for detection of the citrus canker pathogen, *X. campestris* pv. citri (Hartung, 1992). The DNA probes were developed for specific detection of the citrus canker pathogen, *X. campestris* pv. citri which include four different groups. The DNA probes were highly specific for the citrus canker pathogens and did not hybridize to strains of the citrus bacterial spot pathogen, *X. campestris* pv. citrumelo. However, cross-hybridization was observed in strains of *X. campestris* pv. bilvae and *X. campestris* pv. vignicola (Hartung, 1992). Furthermore, a DNA amplification-based procedure was developed for specific and sensitive detection of the citrus canker pathogen based on the DNA sequence of these plasmid probes (Hartung et al., 1993)

CHAPTER 3

OLIGONUCLEOTIDE PRIMERS FOR DETECTION AND IDENTIFICATION OF PLANT PATHOGENIC STRAINS OF *Xanthomonas* BY AMPLIFICATION OF DNA SEQUENCES RELATED TO THE *hrp* GENES OF *Xanthomonas campestris* PV VESICATORIA

The genus *Xanthomonas* Dowson 1939 includes Gram-negative, usually yellow-pigmented bacteria that occur worldwide and cause plant diseases. Over 124 monocotyledonous and 268 dicotyledonous plant species are hosts of *Xanthomonas* (Leyns et al. 1984). Among the species of *Xanthomonas X. campestris* comprises at least 125 different pathovars that are distinguished by the diseases they cause (Bradbury, 1984). The genus *Xanthomonas* also includes strains which may be associated with plant material but are not pathogenic to the plants from which they were isolated (Angeles-Ramos et al., 1991; Gitaitis et al., 1987; Liao and Wells, 1987; Maas et al., 1985). These opportunistic bacteria can be identified as xanthomonads by the presence of xanthomonadins and by unique fatty acid profiles. Although the identification of bacteria in the genus *Xanthomonas* presents no great problem, sub-generic identification of xanthomonads is still difficult.

Traditional methods for the detection and identification of plant pathogenic xanthomonads rely on isolating the organism of interest in pure culture and performing predetermined biochemical, serological and pathological tests (Saettler et al., 1989; Schaad, 1988). Sometimes, non-selective or selective enrichments are required to increase the sensitivity of the isolation, which may be complicated by the presence of fast-growing contaminating bacteria associated with plant tissue (Saettler et al., 1989).

More recently, methods based on metabolic and protein profiling (Chase et al., 1992, Van den Mooter and Swings, 1990; Vauterin et al., 1991ab), and fatty acid analysis (Chase et al., 1992; Yang et al., 1993) have been used for identification, but isolation and purification of the bacterial strain is still required. Polyclonal or monoclonal antisera produced against strains of *X. campestris* have been used for detection and identification, but they have provided variable results. Polyclonal antisera may cross-react with other bacteria and may be unable to differentiate specific strains or pathovars of *X. campestris* (Alvarez and Lou, 1982). Several monoclonal antisera were produced which reacted specifically with all strains of some pathovars of *X. campestris* that infect relatively few genera of hosts (Alvarez et al., 1991, Benedict et al., 1990). However, for certain *X. campestris* pathovars, mainly those that infect several hosts from different genera, no pathovar-specific monoclonal antisera that react with all strains of the respective pathovar have been found (Alvarez et al., 1991, Jones et al., 1993b).

Nucleic acid-based techniques have also been applied for detection and identification of plant pathogenic bacteria (Bereswill et al., 1992, Manulis et al., 1991, Schaad et al., 1989; Seal et al., 1992), including some members of the xanthomonads (Garde and Bender, 1991; Gilbertson et al., 1989, Hartung, 1992; Hartung et al., 1993, Lazo and Gabriel, 1987; Lazo et al., 1987). The techniques developed for detection and identification of xanthomonads were based on random probes (Lazo et al., 1987), or on plasmid DNA fragments specific for a few pathovars of *X. campestris* (Gilbertson et al., 1989; Hartung, 1992; Hartung et al., 1993, Lazo and Gabriel, 1987), or even for a group of strains (Garde and Bender, 1991). Highly conserved regions in the bacterial genome of plant pathogenic bacteria could be more useful for the selection of specific

DNA probes for detection and identification of a larger number of strains, pathovars, or species of *Xanthomonas*.

The *hrp* gene clusters that determine hypersensitivity and pathogenicity may be appropriate for selection of probes for detection and identification of plant pathogenic bacteria. The *hrp* gene cluster is required by bacterial plant pathogens to produce symptoms on susceptible hosts and a hypersensitive reaction (HR) on resistant hosts, or on nonhosts (Willis et al., 1991) and has been found in several plant pathogenic bacteria, such as *Erwinia amylovora* (Beer et al., 1991), *Pseudomonas solanacearum* (Boucher et al., 1987), *P. syringae* pv. phaseolicola (Lindgren et al., 1986), and *X. campestris* pv. vesicatoria (Bonas et al., 1991). Furthermore, *hrp* functions seem to be highly conserved among a number of plant pathogenic bacteria (Ulla Bonas, personal communication, Fenselau et al., 1992, Gough et al., 1992, Hwang et al., 1992). The *hrp* genes of plant pathogenic bacteria are also very similar at the protein level to genes that are involved in the secretion of pathogenicity factors by bacterial pathogens of mammals (Fenselau et al., 1992, Gough et al., 1992). By contrast, nonpathogenic bacteria are unable to produce symptoms on susceptible hosts and HR on nonhosts, apparently because they do not possess DNA sequences similar to *hrp* genes (Lindgren et al., 1986, Stall and Minsavage, 1990). Physically and functionally similar *hrp* sequences occur among several pathovars of *X. campestris*, but not in opportunistic xanthomonads (Bonas et al., 1991, Stall and Minsavage, 1990).

The objective of this study was to examine sequences of the *hrp* genes of *X. campestris* pv. vesicatoria for detection and identification of plant pathogenic xanthomonads. Oligonucleotide primers specific for *hrp* genes were tested for their suitability for identification of these xanthomonads by the polymerase chain reaction

(PCR). Furthermore, the reliability of identification of the xanthomonads to subgeneric classification was assessed by restriction analysis of amplified DNA fragments.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study and their sources are listed in Appendix A. The identity of bacterial strains used here was confirmed by fatty acid analysis (Nancy C. Hodge, personal communication). All strains of *Acidovorax avenae*, *Agrobacterium tumefaciens*, *Erwinia* spp., *Clavibacter michiganense*, *Pseudomonas* spp., *X. campestris*, and *X. maltophilia* were grown on nutrient agar (Becton Dickinson, Cockeysville, MD). Nutrient broth cultures were grown 24 hours on a rotatory shaker (150 rpm) at 28°C. Strains of *X. albilineans* and *X. fragariae* were cultivated on Wilbrink's medium (Koike, 1965). Strains of *Xylella fastidiosa* were grown on PW medium (Davis et al., 1981). Strains of *Escherichia coli* were cultivated on Luria-Bertani medium at 37°C (Miller, 1972). All strains were stored in sterile tap water at room temperature or in 30% glycerol at -70°C, or both. Antibiotics were used to maintain selection for resistance markers at the following final concentrations: ampicillin, 100 µg/ml; tetracycline, 10 µg/ml; rifampicin, 100 µg/ml; and spectinomycin, 50 µg/ml.

Plant material and plant inoculations

All plants were maintained in a growth chamber at 28-30°C during inoculation and incubation. The pepper cultivar Early Calwonder (ECW) and the near-isogenic lines ECW-10R, ECW-20R, and ECW-30R have been described elsewhere (Minsavage et al., 1990). These lines provided a susceptible reaction or a hypersensitive reaction, depending on the strain used.

Fully expanded leaves of plants were inoculated with bacterial suspensions by infiltrating the bacteria into the intercellular spaces with a 1 ml plastic syringe with a 27 gauge needle. The concentration of the inoculum was approximately 5 X 10^8 colony forming units (CFU) per milliliter in sterile tap water, determined by measuring the optical density in a Spectronic 20 spectrophotometer (Bausch and Lomb, Inc., Rochester, NY). Plant reactions were scored over a period of several days.

DNA manipulations

Total genomic DNA was isolated by phenol extraction and ethanol precipitation essentially as described by Ausubel et al. (1987). Plasmid mini-prep preparation of competent cells, ligation, and transformation of *E. coli* cells were performed by standard procedures (Ausubel et al., 1987; Sambrook et al., 1989). For complementation analysis, the helper plasmid pRK2013 was used in triparental mating to mobilize pLAFR3 clones from *E. coli* into *Xanthomonas* cells (Ditta et al., 1980; Figurski and Helinski, 1979).

Hybridization analysis

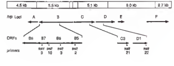
Total genomic DNA and amplified DNA fragments were electrophoresed in 0.7% agarose according to standard procedures (Sambrook et al., 1989). The DNA was then denatured in 0.4 N NaOH and 0.6 M NaCl for 30 min, neutralized for 30 min in 0.5 M Tris Cl and 1.5 M NaCl, pH 7.5, and transferred by the procedure of Southern (1975) to nylon membrane (Schleicher & Schuell, Keene, NH). Southern hybridization and detection of the hybridized DNA was carried out by using the Genius

Nonradioactive DNA labeling and Detection kit (Boehringer Mannheim, Indianapolis, IN) as specified by the manufacturer. Clones containing the desired insert of the *hrp* gene cluster of *X. campestris* pv. vesicatoria, or *in vitro* amplified *hrp* fragments used as probes, were labeled by the random primer (Feinberg and Vogelstein, 1983) incorporation of digoxigenin-labeled deoxyuridine triphosphate (DIG-UTP). Before use, the probes were denatured by boiling for 10 min followed by chilling in an ice ethanol slurry. Hybridization was carried out at 68°C with 0.5X SSC and 0.1% (wt/vol) SDS. The membranes were prewashed twice at room temperature for 5 min in 1X SSC containing 0.1% (wt/vol) SDS. Two final washes were completed at 65 °C for 15 min in 0.5X SSC containing 0.1% (wt/vol) SDS.

DNA amplification

Three sets of oligonucleotide primers were selected from the nucleotide sequence of the *hrp* region of *X. campestris* pv. vesicatoria (Ulla Bonas, personal communication). Primers RST2 (5'AGGCCCTGGAAGGTGCCCTGGA3') and RST3 (5'ATCGCACTGCGTACCGCGCGCGA3') delineated a 840 bp fragment, RST9 (5'GGCACTATGCAATGACTG3') and RST10 (5'AATACGCTGGAACTGCTG3') delineated a 355-bp fragment, and RST21 (5'GCACGCTCCAGATCAGCATCGAGG3') and RST22 (5'GGCATCTGCATGCGTGCTCTCCGA3') delineated a 1,075-bp fragment. The primers map to the complementation groups *hrpB*, *hrpC*, and *hrpD* of *X. campestris* pv. vesicatoria (Fig. 3-1). Furthermore, the sequences of the oligonucleotide primers RST3 and RST9 originate from the *hrpB6*, a gene for a putative ATPase that seems to be highly conserved among different bacteria at the protein sequence level (Fenselau et al., 1992). Oligonucleotide primers were synthesized with a model 394 DNA Synthesizer

Fig. 3.1. Structural organization of the *hrp* region in *Xanthomonas campestris* pv. vesicatoria. (A) *Eco*RI fragments of the *hrp* region. (B) Position and orientation of the *hrp* loci, designated *hrpA* to *hrpF* (Bonas *et al.*, 1991, Schulte and Bonas, 1992). (C) Position and orientation of the open reading frames (ORFs). The sizes of the loci are based on a combination of genetic and sequence analysis from which possible open reading frames (ORF) are predicted (Ulla Bonas, personal communication). Only the open reading frames relevant for this study, *hrpB5* to *hrpB8*, *hrpC3*, and *hrpD*, are shown here. For each RST oligonucleotide primer used for DNA amplification, its position in the DNA sequence is indicated by an asterisk (*).


A
4.5 kb
5.5 kb
5.1 kb
8.0 kb
2.7 kb
B
hrp Loci
A
B
C
D
E
F
C
ORFs
B8
B7
B6
B5
C3
D1
primers
RST 9
RST 10
RST 3
RST 2
RST 21
RST 22

(Applied Biosystems, Foster City, CA) by the DNA Synthesis Laboratory, University of Florida, Gainesville.

DNA was amplified in a total volume of 50 µl. The reaction mixture contained 5 µl of 10X buffer (500 mM KCl, 100 mM Tris Cl [pH 9.0 at 25°C], 1% Triton X-100), 1.5 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (Boehringer Mannheim), 25 pmol of each primer, and 1.25 units of *Taq* polymerase (Promega, Madison, WI). The amount of template DNA added was 100 ng of purified total bacterial DNA or 25 ng of a plasmid preparation, unless otherwise stated. The reaction mixture was covered with 50 µl of light mineral oil. A total of 30 amplification cycles were performed in an automated thermocycler PT 100 60 (MJ Research, Watertown, MA). Each cycle consisted of 30 s of denaturation at 95°C, 30 s of annealing at 62°C, and 45 s of extension at 72°C for the primers RST2 and RST3; 30 s at 95°C, 30 s at 52°C and 45 s at 72°C for the primers RST9 and RST10, and 30 s at 95°C, 40 s at 61°C and 45 s at 72°C, for the primers RST31 and RST22. The last extension step was extended to 5 min. Amplified DNAs were detected by electrophoresis in 0.9% agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.2) at 5 V/cm of gel (Sambrook et al., 1989). The gel was stained with 0.5 µg of ethidium bromide per ml and then photographed over a UV transilluminator (Fotodyne Inc., New Berlin, WI) with type 55 Polaroid film (Polaroid, Cambridge, MA).

Restriction endonuclease analysis

The DNA fragments amplified from different bacterial strains were restricted with the frequent cutting endonucleases *Cfo*I, *Hae*III, *Sau*3AI, or *Taq*I, according to conditions specified by the manufacturer (Promega). The restricted fragments were separated by electrophoresis in 4% agarose gels (3% NuSieve and 1% Seakem GTG

[FMC BioProducts, Rockland, ME]) in TAE buffer at 8 V/cm. Phage λ *Pst*I restricted DNA fragments were used as molecular standards. The gel was stained with 0.5 µg of ethidium bromide per ml for 40 min and then destained in 1 mM $MgSO_4$ for 1 hr and photographed over a UV transilluminator with type 55 Polaroid film.

Results

Specificity of the oligonucleotide primers to the *hrp* gene cluster

Three pairs of oligonucleotide primers were tested for amplification of fragments from genomic DNA of strain 75-3 of *X. campestris* pv. vesicatoria and from plasmids that contain cloned parts of the *hrp* region from strain 75-3. Plasmid pXV9 harbors a fragment of approximately 27 kb of strain 75-3 of *X. campestris* pv. vesicatoria containing almost the entire *hrp* region (Bonas et al., 1991). Plasmid pXV5.5 harbors a 5.5 kb EcoRI fragment containing part of the *hrp* complementation groups *hrpA* and *hrpB* (Fig. 3-1). The 5.1 kb EcoRI insert of plasmid pXV5.1 maps to the complementation groups *hrpC* and *hrpD* (Fig. 3-1). We amplified fragments of the expected 355, 840, and 1,075 bp in length from total genomic DNA of strain 75-3 of *X. campestris* pv. vesicatoria by using primers RST9 and RST10, RST2 and RST3, and RST21 and RST22, respectively (Fig. 3-2). The 355- and 840-bp fragments were also amplified from plasmids pXV9 and pXV5.5 (Fig. 3-2), whereas the 1,075-bp fragment was amplified from plasmids pXV9 and pXV5.1 (Fig. 3-2), thus confirming the sizes and locations of fragments predicted by the DNA sequence analysis (Ulla Bonas, personal communication). The 355- and 840-bp sequences correspond to genes in the *hrpB* operon, whereas the 1075 bp fragment occurs in the *hrpC*/*hrpD* region (Fig. 3-1). Except for *hrpB6*, which encoded a putative ATPase (Fenselau et al., 1992), the

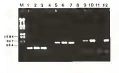


Fig 3.2 Amplification of fragments of the *hrp* gene cluster from *Xanthomonas campestris* pv vesicatoria 75-3 The following DNAs were used 75-3 (lanes 1, 5 and 9), plasmid pXV9 (lanes 2, 6 and 10), plasmid pXV5.5 (lanes 3, 7, and 11), and plasmid pXV5.1 (lanes 4, 8 and 12) Lanes M, phage λ restricted with *Eco*RI and *Hind*III 1 to 4 amplification with primers RST9 and RST10 5 to 8, amplification with primers RST2 and RST3 9 to 12 amplification with primers RST21 and RST22 Molecular sizes are given in base pairs

functions of these genes are unknown. The *hrpD1* gene is highly similar to a sequence of pathogenicity genes in *X. campestris* pv. glycines (Ulla Bonas, personal communication, Hwang et al., 1992).

The identity of the amplified fragments was further confirmed by restriction enzyme analysis. The 355-, 840- and 1,075-bp fragments amplified from both total DNA of strain 75-3 of *X. campestris* pv. vesicatoria and from DNA of pXV9, pXV5.1, and pXV5.5 were digested with *Cfo*I, HaeIII, Sau3AI, and *Taq*I. The banding patterns were identical for each of the three sets of fragments amplified from strain 75-3 and plasmids containing cloned parts of the *hrp* region (Fig. 3-3) and matched the restriction map generated from the DNA sequence of the *hrp* gene cluster of *X. campestris* pv. vesicatoria 75-3 (Ulla Bonas, personal communication).

Similarity of the *hrp* fragments amplified from *X. campestris* pv. vesicatoria to DNA of other bacteria

Total genomic DNA of strains of different pathovars of *X. campestris* and of *Xanthomonas* spp. was digested with EcoRI, separated in agarose gels, blotted, and probed with each of the three fragments amplified from DNA of *X. campestris* pv. vesicatoria strain 75-3. The hybridization signals in the genomic DNA of *X. campestris* pv. vesicatoria 75-3 corresponded to the predicted 7.4 kb *Bam*HI and 5.5 kb EcoRI fragments (355- and 840-bp probes) and to the 6.0 kb *Bam*HI and 5.1 kb EcoRI fragments (1,075-bp probe) (Fig. 3-4). Homology to these three *hrp* fragments of *X. campestris* pv. vesicatoria strain 75-3 was detected in strains of *X. fragariae* and of 28 different pathovars of *X. campestris* (Fig. 3-4, Table 3-1). However, polymorphisms were observed in the DNA from these different pathovars (Fig. 3-4). Total genomic DNA of *X. albilineans*, *X. campestris* pv. secalis, and *X. campestris* pv. translucens, which are pathogens of monocotyledonous plants, as well as DNA of the non plant

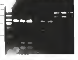


Fig 3 3 *Taq*I restriction endonuclease analysis of fragments of the *hrp* gene cluster amplified from total genomic DNA from *Xanthomonas campestris* pv vesicatoria 75 3 and from plasmids containing the *hrp* region. Lanes M, phage λ restricted with *Pst*I; 1 to 3 the 355 bp fragments from *X campestris* pv vesicatoria 75 3 and plasmids pXV9 and pXV5 5 respectively lanes 4 to 6 the 840-bp fragments from *X. campestris* pv vesicatoria 75 3 and plasmids pXV9 and pXV5 5. respectively lanes 7 to 9, the 1 075-bp fragments from *X campestris* pv vesicatoria 75 3 and plasmids pXV9 and pXV5 1, respectively Molecular sizes are given in base pairs

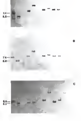


Fig 3-4 Hybridization of the *hrp* fragments amplified from *Xanthomonas campestris* pv vesicatoria 75 3 to total genomic DNA of strains of *Xanthomonas campestris* Approximately 3 µg of *Eco*RI-digested DNA was loaded per lane. The blots were probed with the labeled 355-bp (A), 840-bp (B) and 1 075-bp (C) fragments amplified from *X campestris* pv vesicatoria 75-3 Lanes: 1, *X campestris* pv vesicatoria 75 3 *Bam*HI digested, 2 *X campestris* pv vesicatoria 75 3, 3, *X campestris* pv vesicatoria XV56, 4 *X campestris* pv alfalfae KS, 5, *X campestris* pv begoniae XCB9 6, *X campestris* pv campestris 33913 7 *X campestris* pv glycines 87-2, 8 *X campestris* pv holcicola G-23 9, *X campestris* pv malvacearum RIATC 10, *X campestris* pv phaseoli 85-6, 11, *X campestris* pv pruni FLA1, 12, *X campestris* pv secalis XC129C, 13 *X campestris* pv translucens 80-1 Molecular sizes are given in kilobase pairs.

Table 3-1 Hybridization of the *hrp* fragments amplified from *Xanthomonas campestris* pv. vesicatoria 75-3 to total genome DNA and amplification of *hrp*-related fragments from different bacterial strains

Strain	Southern hybridization			DNA amplification		
	355 bp	840 bp	1,075 bp	355 bp	840 bp	1,075 bp
Xanthomonas						
X. campestris						
pv. alfalfae KS	+	+[a]	+	(+)	+	+
pv. armoraciae 63-27	+	+	+		+	+
pv. bilvae XCB	+	+	+		+	+
pv. begoniae XC89	+	+	+		+	+
pv. campestris 33913	+	+	+	—	+	+
pv. carotae #13	+	+	+		(+)	+
pv. citri 9771	+	+	+	—	+	+
pv. citrumelo F1	+	+	+	(+)	+	+
pv. dieffenbachiae 729	+	+	+	—	+	+
pv. fici X151	+	+	+	+	+	+
pv. gardneri XG101	+	+	+	—	(+)	+
pv. glycines 87 2	+	+	+	—	+	+
pv. holcicola G 23	+	+	+	—	-	+
pv. incanae 9561 1	+	+	+		+	+
pv. macculifoliigardeniae X22j	+	+	+	(+)	+	+
pv. malvacearum RIATC	+	+	+		+	+
pv. manihotis Xm125D	+	+	+	(+)	+	+
pv. papavericola XP5	+	+	+		(+)	+
pv. phaseoli 85-6	+	+	+		+	+
pv. phaseoli "fuscans" XP163A	+	+	+		+	+
pv. physalidicola XP172	+	+	+	+	+	+
pv. pelargonii XCP58	+	+	+		(+)	+
pv. poinsettiicola 071-424	+	+	+		+	+
pv. pruni X1219L	+	+	+		+	+
pv. raphani 69-2	+	+	+	—	+	+
pv. secalis XC129C						
pv. taraxaci XT11A	+	+	+	—	(+)	+
pv. translucens 80 1				—		
pv. vesicatoria						
75 3	+	+	+	+	+	+
XV56		+	+	—	+	+
pv. vignicola 81 30	+	+	+		+	+
pv. vitians XV1T	+	+	+	—	+	+
X198	+	+	+	+	+	+
XCF	+	+	+	(+)	+	+
T 55						
INA				—	—	—

Continued on following page

Table 3-1—*Continued*

Strain	Southern hybridization			DNA amplification		
	355 bp	840 bp	1,075 bp	355 bp	840 bp	1,075 bp
X. albilineans 91-065	−			−	−	−
X. fragariae X1297	+	+	+		+	+
X. maltophilia	+	+	+	−	−	−
Acidovorax avenae						
subsp. *avenae* UK142-A	−		+	−	−	−
subsp. *citrulli* UK20			+	−		
Agrobacterium tumefaciens LBA 1050		+	+			
Clavibacter michiganense subsp. *michiganensis* 69-1				−	−	−
Erwinia						
E. carotovora subsp. *carotovora*						
K-SR 347		+	+			
B-SR 38	+	+	+	−	−	−
E. herbicola NF 33					−	
E. stewartii SW2		−	+	−	−	−
Pseudomonas						
P. solanacearum K60	−	−				
P. syringae						
pv. *syringae* INB						
pv. *tomato* 987						
Xylella fastidiosa 89-1				−		

[a]+, positive reaction; −, negative reaction; (+), weak signal

pathogens *X. maltophilia* and the opportunistic strains of *X. campestris* T-55 and INA, did not hybridize to any of the three *hrp* fragments amplified from *X. campestris* pv. vesicatoria 75-3 (Fig. 3-4, Table 3-1).

DNA from strains of the plant pathogens of the genera *Acidovorax*, *Agrobacterium*, *Clavibacter*, *Erwinia*, *Pseudomonas*, and *Xylella* failed to hybridize to the three *hrp* fragments (Table 3-1). Furthermore, total genomic DNA of *E. herbicola*, a bacterium commonly associated with plant tissue, also did not hybridize to any of the *hrp* fragments amplified from *X. campestris* pv. vesicatoria 75-3 under the conditions used (Table 3-3).

Amplification of the *hrp* fragments from other *X. campestris* pathovars and related *Xanthomonas* spp.

Primer pairs RST2 plus RST3 and RST21 plus RST22 were used to amplify DNA sequences from strains representing *X. fragariae* and 28 different pathovars of *X. campestris*. In all cases with both sets of primers, fragments of identical sizes were amplified from different pathovars of *X. campestris* and related *Xanthomonas* spp. (Fig. 3-5, Table 3-1). However, amplification with the primers RST2 and RST3 usually gave low yield of DNA for strains of *X. campestris* pv. carotae, *X. campestris* pv. gardneri, *X. campestris* pv. papavericola, *X. campestris* pv. pelargonii, and *X. campestris* pv. taraxaci (Fig. 3-5, Table 3-1). Although DNA isolated from *X. campestris* pv. holcicola hybridized to the 840 bp *hrp* fragment from *X. campestris* pv. vesicatoria strain 75-3 (Fig. 3-4), primers RST2 and RST3 did not amplify the DNA fragment from this pathovar (Table 3-1). No amplification occurred with purified total genomic DNA from a number of bacteria, including plant pathogenic strains of the xanthomonads *X. albilineans*, *X. campestris* pv. secalis, and *X. campestris* pv. translucens, and of the genera *Acidovorax*, *Agrobacterium*, *Clavibacter*, *Erwinia*,

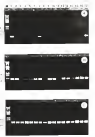


Fig. 3-5 Amplification of the 355-bp (A), 840-bp (B), and 1,075-bp (C) fragments of the *hrp* gene cluster from strains of *Xanthomonas campestris*. Lanes: M, phage λ restricted with *Eco*RI and *Hind*III, 1, *X. campestris* pv. vesicatoria 75-3, 2, *X. campestris* pv. bilvae XCB, 3, *X. campestris* pv. carotae #13, 4, *X. campestris* pv. citri 9771, 5, *X. campestris* pv. citrumelo F1, 6, *X. campestris* pv. dieffenbachiae 729, 7, *X. campestris* pv. fici X151, 8, *X. campestris* pv. gardneri XG101, 9, *X. campestris* pv. maculifoliigardeniae X22j, 10, *X. campestris* pv. manihotis Xm125D, 11, *X. campestris* pv. pelargonii XCP58, 12, *X. campestris* pv. phaseoli "fuscans" XP163A, 13, *X. campestris* pv. poinsettiicola 071-424, 14, *X. campestris* pv. taraxaci XT11A, 15, *X. campestris* pv. vignicola 81-30, 16, *X. campestris* pv. vitians XVIT, 17, *X. campestris* pv. physalidicola XP172. Molecular sizes are given in base pairs.

Pseudomonas, and *Xylella* and from the non plant pathogens *E. herbicola*, *X. maltophilia*, and the opportunistic strains of *X. campestris* T-55 and INA, when either RST2 plus RST3 or RST21 plus RST22 were used (Table 3-1). The failure to amplify the DNA fragments from all those bacterial strains was expected because of the lack of hybridization to the three *hrp* fragments from *X. campestris* pv. vesicatoria 75-3 (Table 3-1).

DNA fragments delineated by the primers RST9 and RST10 were consistently amplified only from strains of *X. campestris* pv. fici, *X. campestris* pv. physalidicola, *X. campestris* pv. vesicatoria, and *X. campestris* X19E (Fig. 3-5, Table 3-1). However, some pathovars of *X. campestris*, including alfalfae, citrumelo, maculifoliigardiniae, and manihotis, as well as the strain XCF, sometimes produced low yield in the amplification of the 355 bp fragment (Fig. 3-5, Table 3-1).

The identity of these *hrp* related fragments amplified from different strains of plant pathogenic *Xanthomonas* spp. was further confirmed by Southern hybridization analysis. Internal portions of the 840- and 1 075-bp DNA fragments, as well as the entire 355 bp fragment amplified from *X. campestris* pv. vesicatoria 75-3, were used as probes. The internal probe for the 840 bp fragment consisted of a 271-bp insert of the plasmid pXV840, and the internal probe for the 1 075-bp fragment consisted of a 335-bp insert of the plasmid pXV1075. The inserts were obtained from fragments amplified from DNA of *X. campestris* pv. vesicatoria strain 75-3 by cloning *Sau*3AI digests into the *Bam*HI site of the vector pBluescript II KS +/- (Stratagene, La Jolla, CA). In all cases, the DNA fragments amplified from different strains of *Xanthomonas* spp. with each set of primers hybridized with the respective probe (data not shown).

Restriction endonuclease analysis of amplified *hrp*-related DNA fragments

To address the question of degree of sequence conservation among different strains, I examined the 840- and 1,075-bp *hrp* fragments amplified from strains of different pathovars of *X. campestris*, as well as from *X. fragariae*, by restriction endonuclease analysis with the endonucleases *Cfo*I, *Hae*III, *Sau*3AI, and *Taq*I. Restriction fragment length polymorphisms were apparent for both fragments. For example, the 1,075-bp fragment, amplified from strains of different pathovars of *X. campestris* by using primers RST21 and RST22 and then restricted with *Hae*III and *Sau*3AI, yielded different restriction patterns (Fig. 3-6). Although RFLPs were observed with all four endonucleases for both the 840- and the 1,075-bp fragments, restriction analysis with *Cfo*I and *Hae*III produced more distinct patterns for differentiation of the groups or pathovars of *X. campestris*.

Pathogenicity function of the DNA sequence of *X. campestris* pathovars from which *hrp* related fragments were amplified

To determine if the DNA sequence from which *hrp* related fragments were amplified has common functions in different strains of *X. campestris*, I investigated the functional homology of these fragments in *X. campestris* pv. vesicatoria group B and *X. campestris* pv. pelargonii. The cosmid clones pXV56/3-48, of a pLAFR3 library of strain XV56 of *X. campestris* pv. vesicatoria group B (Beaulieu et al., 1991), and pXCP58/2, of a pLAFR3 library of *X. campestris* pv. pelargonii XCP58 (Gerald V. Minsavage, personal communication), were identified by amplification of the 1,075-bp *hrp* fragment with the primers RST21 and RST22. Plasmid pXV56/3-48 was transferred into the *X. campestris* pv. vesicatoria mutants 85-10::*hrpA22*, 85-10::*hrpB85*, 85-10::*hrpC44*, 85-10::*hrpD137*, 85-10::*hrpE75*, and 85-10::*hrpF318*, which carry Tn3-*gus* insertions in the different *hrp* complementation groups (Bonas et

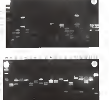


Fig. 3-6 Restriction analysis of the 1,075-bp DNA fragment of the *hrp* gene cluster amplified from strains of *Xanthomonas campestris* and restricted with the endonucleases *Hae*III (A) and *Sau*3AI (B). Lanes: M, phage λ restricted with *Pst*I; 1, *X. campestris* pv. vesicatoria 75-3; 2, *X. campestris* pv. malvae XCB; 3, *X. campestris* pv. carotae #13; 4, *X. campestris* pv. citri 9771; 5, *X. campestris* pv. citrumelo F1; 6, *X. campestris* pv. dieffenbachiae 729; 7, *X. campestris* from *Strelitzia reginae* strain X198; 8, *X. campestris* pv. fici X151; 9, *X. campestris* pv. maculifoliigardeniae X22j; 10, *X. campestris* pv. manihotis Xm125D; 11, *X. campestris* pv. pelargonii XCP58; 12, *X. campestris* pv. phaseoli "fuscans" XP163A; 13, *X. campestris* pv. poinsettiicola 071-424; 14, *X. campestris* pv. taraxaci XT11A; 15, *X. campestris* pv. vignicola 81-30; 16, *X. campestris* pv. vitians XVIT; 17, *X. campestris* pv. papavericola XP5; 18, *X. campestris* pv. holcicola G-23. Molecular sizes are given in base pairs.

al., 1991). Plasmid pXV56/3-48 fully restored the pathogenicity of mutants with mutations in *hrpB*, *hrpD*, and *hrpE*, and the hypersensitive reaction-inducing ability but not pathogenicity to the *hrpC* mutant. However, this plasmid failed to complement the *hrpA* and *hrpF* mutants. Similarly, plasmid pXCP58/2 from *X. campestris* pv. pelargonii was also transferred into the six nonpathogenic Tn*3-gus* mutants of *X. campestris* pv. vesicatoria 85-10. This plasmid fully restored the wild type phenotype to mutants with mutations in *hrpC*, *hrpD*, and *hrpE* but failed to complement *hrpA*, *hrpB*, and *hrpF* Tn*3-gus* mutants.

Sensitivity of the amplification of *hrp* fragment for detection of *X. campestris* pv. vesicatoria

The sensitivity of the amplification of specific DNA fragments in detection of *X. campestris* was determined by using 10-fold dilutions of purified total bacterial DNA of *X. campestris* pv. vesicatoria 75-3. The oligonucleotide primers RST9 plus RST10 and RST2 plus RST3 were used for amplification of the 355- and 840-bp *hrp*, respectively, from samples containing as little as 0.25 pg of total bacterial DNA after 30 cycles of DNA amplification (Fig. 3.7).

Discussion

Sequence homology to small *hrp* fragments amplified from *X. campestris* pv. vesicatoria 75-3 was found among plant pathogenic strains of several pathovars of *X. campestris* and related *Xanthomonas* spp. by Southern hybridization analysis. DNA probes representing regions of the *hrpB*, *hrpC*, and *hrpD* loci hybridized strongly to total DNA from strains of 28 different pathovars of *X. campestris*. These results confirm and extend previous observations (Bonas et al., 1991; Stall and Minsavage,

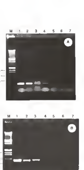


Fig. 3-7 Amplification of the 355-bp (A) and (B) 840-bp fragments of complementation group B of the *hrp* gene cluster from samples with different amounts of DNA template of *Xanthomonas campestris* pv. vesicatoria 75-3. Lanes: M, phage λ restricted with *Eco*RI and *Hind*III; 1, 25 ng; 2, 2.5 ng; 3, 0.25 ng; 4, 25 pg; 5, 2.5 pg; 6, 0.25 pg; 7, 0.025 pg. Molecular sizes are given in base pairs.

1990) on the conservation of the *hrp* region among the plant pathogenic xanthomonads. In contrast, the DNA of *X. campestris* pv. secalis and *X. campestris* pv. translucens did not hybridize to the *hrp* fragments. *X. campestris* pv. secalis is genetically only weakly related to a few pathovars of *X. campestris* based on DNA-DNA hybridization experiments (Hildebrand et al., 1990). Previously, *X. campestris* pv. translucens has shown only weak hybridization to DNA probes representing the entire *hrp* gene cluster region of *X. campestris* pv. vesicatoria (Bonas et al., 1991; Stall and Minsavage, 1990). Although hybridization to DNA of all strains of *X. campestris* included in this study was not observed, the *hrp* region seems useful for detection and identification of a large number of plant pathogenic xanthomonads. A major advantage in using *hrp* sequence is the lack of homology to DNA of non plant pathogenic xanthomonads, as observed for *X. maltophilia* and the opportunistic *X. campestris* strains T-55 and INA, as well as other plant pathogens of the genera *Acidovorax*, *Agrobacterium*, *Clavibacter*, *Erwinia*, *Pseudomonas*, and *Xylella*.

The three pairs of oligonucleotide primers described in this study are specific for the *hrpB*, *hrpC*, and *hrpD* regions of *X. campestris* pv. vesicatoria 75-3 and were used to amplify homologous DNA fragments from *X. fragariae* and from 31 of 33 plant pathogenic taxa of *X. campestris* tested, which comprise at least 28 different pathovars of this species. In all cases, each set of primers amplified DNA fragments identical in size, suggesting a high degree of structural conservation between operons, as seen with primers RST21 and RST22. Cloned regions of DNA of *X. campestris* pv. vesicatoria group B and *X. campestris* pv. pelargonii, from which the *hrp* related fragments were also amplified, fully restored pathogenicity to several nonpathogenic Tn3-gus mutants of *X. campestris* pv. vesicatoria 85-10. This supports the contention that these fragments were amplified from DNA sequences which also control the pathogenicity in other xanthomonads.

In contrast to the narrow spectrum of oligonucleotide primers previously used for detection and identification of only certain strains of *X. campestris* (Hartung et al., 1993), the *hrp* specific primers RST2 plus RST3 and RST21 plus RST22 seem very useful for the identification of a large range of plant pathogenic xanthomonads. This is perhaps not surprising, because the *hrp* region seems very conserved among different plant pathogenic xanthomonads as determined by Southern hybridization experiments in the present and previous studies (Bonas et al., 1991; Stall and Minsavage, 1990). Furthermore, the nucleotide sequences of the primers RST21 and RST22 are identical to corresponding sequences of pathogenicity genes of *X. campestris* pv. glycines (Hwang et al., 1992). In addition, the cloned fragment from *X. campestris* pv. glycines complements *hrpD* mutants of *X. campestris* pv. vesicatoria (Ulla Bonas, personal communication), suggesting functional homology between these regions from both pathovars.

Primers RST9 and RST10, which delineate a fragment of 355 bp, allowed DNA amplification only from a limited number of pathovars of *X. campestris*, despite hybridization of the fragment to the majority of the strains of this species. It should be noted that the sequence of RST9 originates from *hrpB6*, a gene for a putative ATPase that seems to be highly conserved among different bacteria at the protein sequence level (Fenselau et al., 1992). These results indicate differences in the DNA sequences of *Xanthomonas* spp. corresponding to one or both primers used. However, that set of primers seems useful for specific detection of strains of *X. campestris* pv. vesicatoria group A, *X. campestris* pv. fici, *X. campestris* pv. physalidicola, and *X. campestris* X198.

The identification of strains of *X campestris* at the pathovar level is difficult even by using different techniques, such as fatty acid analysis (Chase et al , 1992, Graham et al. 1990), serology (Alvarez et al , 1991), metabolic profile (Chase et al., 1992 Van den Mooter and Swings 1990) and SDS-PAGE of proteins (Vauterin et al , 1991a), thus, the restriction analysis of amplified *hrp* related fragments may be a valuable tool for identification of subgroups of plant pathogenic strains and pathovars of *X campestris* For example, restriction analysis with frequent-cutting endonucleases produced a characteristic restriction pattern for the 840- or 1 075-bp DNA fragments amplified from the *Xanthomonas* spp included in this work, this appears to be highly conserved within each group of certain plant pathogenic xanthomonads (Chapter 4) In this way RFLP profiles of a particular *hrp* region could be established for each plant pathogenic group of xanthomonads thus facilitating the identification and classification of these bacterial strains

The use of oligonucleotide primers provides a sensitive and specific tool for detection of DNA by amplification. Theoretically, the limit of detection of an amplifiable DNA sequence is estimated to be as low as one single target cell in the reaction mixture (Steffan and Atlas 1991) In our studies, we were able to find a detectable signal for as low as 0 25 pg of total bacterial DNA. This level of sensitivity is comparable to those obtained by others without the use of any technique to enhance the signal (Pickup, 1991, Steffan and Atlas, 1988). Furthermore, *X campestris* pv vesicatoria could be detected in plant samples containing less than 100 CFU/ml, without prior enrichment or cultivation of the organism (Chapter 7) In addition to the sensitivity of the technique, the specificity of the oligonucleotide primers to plant pathogenic xanthomonads certainly assures selectivity against background nontarget microorganisms which are always present in the samples

In conclusion, the results presented here indicate that plant pathogenic strains of *X. campestris* and related xanthomonad species can be detected and may be identified by analysis of DNA fragments amplified with *hrp* gene-specific primers. The conservation of the *hrp* DNA sequence among a large number of pathovars of *X. campestris*, as well as in related *Xanthomonas* spp., but lack of the *hrp* DNA sequence among non-plant pathogenic bacteria, makes this method a useful tool for detection and identification of many plant pathogens. Consequently, *hrp* oligonucleotide primers may be also useful to determine the pathogenic nature of unknown xanthomonads. This is particularly significant for assessing the complex population of plant pathogenic and nonpathogenic xanthomonads associated with plants and plant parts. The presence of plant pathogenic strains in such samples may be determined by amplification of the *hrp* fragments without the need of the troublesome methods of isolation of the organism and inoculation into potential host plants. Moreover, RFLPs detected in the genome of different strains seem valuable for the study of the relatedness of plant pathogenic xanthomonads, particularly among *Xanthomonas* spp. and pathovars of *X. campestris*. Of course, this has to be extended by testing larger numbers of strains for each species, subspecies, or pathovar. The genetic methods of analyzing populations of bacteria will provide valuable additional information for taxonomic, ecological, and epidemiological studies of plant pathogenic xanthomonads.

CHAPTER 4
CHARACTERIZATION OF PLANT PATHOGENIC *Xanthomonas* BASED ON RESTRICTION ANALYSIS OF AMPLIFIED DNA SEQUENCES RELATED TO THE *hrp* GENES

Identification of plant pathogenic xanthomonads has relied on isolating the organism of interest in pure culture and performing predetermined biochemical, serological, and pathological tests (Bradbury, 1984; Saettler et al., 1989; Schaad, 1988). More recently, methods based on metabolic fingerprinting (Chase et al., 1992; Hildebrand et al., 1993; Jones et al., 1993a; Van den Mooter and Swings, 1990; Vermeire et al., 1993), protein profiling (Vauterin et al., 1991ab), and fatty acid composition (Chase et al., 1992; Hodge et al., 1992; Yang et al., 1993) have also been used for identification of these plant pathogens. Although these methods have proved useful for the differentiation of certain plant pathogenic xanthomonads, the specific identification of the xanthomonads at the subgeneric level is still difficult. Further, the difficulty is particularly evident for the infrasubspecific identification of the members of *X. campestris*. This species includes at least 125 different pathovars which are usually distinguished only on the basis of their host range or by the disease they cause (Bradbury, 1984; Dye et al., 1980; Hayward, 1993). Therefore, there is a need for more rapid and unambiguous procedures for identification and detection of these plant pathogens.

In recent years, nucleic acid-based techniques have been used for characterization of the xanthomonads. DNA-DNA hybridizations were applied to determine the intrageneric relationships among different xanthomonads (Hildebrand et al., 1990, Palleroni et al., 1993, Vauterin et al., 1993) and 21 DNA homology groups were established (Vauterin et al., 1993). Some DNA homology groups were delineated which include pathovars of *X. campestris* causing diseases on closely related plants as occurs with the xanthomonads pathogenic on leguminous (Hildebrand et al., 1990, Palleroni et al., 1993, Vauterin et al., 1993), gramineous (Vauterin et al., 1993), and cruciferous plants (Vauterin et al., 1993). Furthermore, these groups established based on DNA homology are strongly supported by the groupings based on SDS-PAGE of proteins and fatty acid analyses (Vauterin et al., 1991a, 1992, Yang et al., 1993). However, the genetic relatedness among the xanthomonads is not always correlated with pathogenicity features.

A striking feature of the plant pathogenic xanthomonads is the genetic diversity among strains of the same pathovar or different pathovars of *X. campestris* that cause diseases on the same or highly related hosts. For example, strains of *X. campestris* pv. vesicatoria groups A and B that cause diseases on solanaceous plants are only about 33% related on the basis of DNA homology (Stall et al., 1994). Further, this low genetic similarity and the differences in phenotypic features strongly supports the conclusion that these two groups of *X. campestris* pv. vesicatoria may even belong to different species (Stall et al., 1994). In another study, Egel et al. (1991) examined the genetic relatedness of the xanthomonads causing diseases of citrus and found that strains of the citrus canker pathogen *X. campestris* pv. citri and strains of the citrus bacterial spot agent *X. campestris* pv. citrumelo are less than 60% related to one another. The diverse nature of the *X. campestris* strains causing diseases on citrus has also been determined on the basis of genomic fingerprinting (Hartung and Civerolo, 1987), analysis of restriction fragment length polymorphism (RFLP) (Gabriel et al., 1988, 1989, Graham et al., 1990, Hartung and Civerolo 1989) and fatty acid composition (Graham et al., 1990, Stall and Hodge, 1989).

The genetic diversity of the xanthomonads has also been investigated by using other nucleic acid-based techniques, such as genomic fingerprinting with frequent and rare-cutting restriction endonucleases (Egel et al., 1991, Hartung and Civerolo 1987, Pruvost et al., 1992) and RFLP analyses of plasmid and genomic DNAs (Berthier et al., 1993, DeParasis and Roth, 1990 Gabriel et al., 1988, 1989; Garde and Bender, 1991, Gilbertson et al., 1989 Gottwald et al., 1991 Graham et al., 1990; Hartung 1992, Hartung and Civerolo 1989, 1991, Lazo and Gabriel, 1987, Lazo et al., 1987, Leach et al., 1990 1992, Qhobela and Claflin, 1988 1992 Verdier et al. 1993). In an extensive study, Lazo et al. (1987) differentiated strains representing 26 different pathovars of *X campestris* based on RFLP analysis by using random DNA probes obtained from a cloned fragment derived from *X. campestris* pv. citrumelo. The RFLP analyses revealed profiles highly conserved and characteristic for each pathovar tested. It was possible to differentiate all the pathovars of *X. campestris* included in the study by using more than one DNA probe or by digesting the genomic DNA with different restriction endonucleases (Lazo et al. 1987). Plasmid DNA fragments have also been examined as probes for specific identification of certain groups of *X. campestris* (Garde and Bender, 1991, Gilbertson et al., 1989, Hartung, 1992, Hartung et al., 1993, Lazo and Gabriel 1987). However, DNA probes derived from specific regions in the bacterial genome and conserved among plant pathogenic bacteria may be more meaningful for specific differentiation and characterization of strains, pathovars, or species of *Xanthomonas*.

The hypersensitive reaction and pathogenicity (*hrp*) gene cluster that has been found in several plant pathogenic bacteria may be appropriate for specific identification of plant pathogenic xanthomonads. The *hrp* genes are required by plant pathogens to cause disease on susceptible hosts and hypersensitive reaction (HR) on resistant, or on nonhost plants (Willis et al., 1991). The *hrp* genes have been found in different plant pathogenic bacteria, including *Erwinia amylovora* (Beer et al., 1991), *Pseudomonas solanacearum* (Boucher et al. 1987), *P. syringae* pv. phaseolicola (Lindgren et al., 1986), and *X. campestris* pv. vesicatoria (Bonas et al., 1991). Furthermore, the *hrp* gene sequence seems to be highly conserved among different pathogenic bacteria at the functional level (Ulla Bonas, personal communication; Fenselau et al., 1992; Gough et al., 1992; Hwang et al., 1992). On the contrary, nonpathogenic bacteria are unable to cause disease or HR on plants and they apparently lack DNA sequences similar to the *hrp* genes (Lindgren et al., 1986; Stall and Minsavage, 1990). In previous studies, the *hrp*-genes sequences were useful for differentiating pathogenic from nonpathogenic xanthomonads (Stall and Minsavage, 1990), as well as for differentiating strains from different pathovars of *X. campestris* and related plant pathogenic *Xanthomonas* spp. (Chapter 3).

The purpose of this study was to characterize the plant pathogenic xanthomonads by restriction analysis of DNA sequences related to the *hrp* genes of *X. campestris* pv. vesicatoria. I used oligonucleotide primers specific for the *hrp* genes to amplify *hrp*-related DNA fragments from different plant pathogenic xanthomonads by the polymerase chain reaction. Further, the reliability of the identification of the xanthomonads was assessed by examining different groups of plant pathogenic xanthomonads that comprise the species *X. albilineans*, *X. fragariae*, and at least 31 different pathovars of *X. campestris*.

Materials and Methods

Bacterial strains and culture conditions

The bacterial strains used in this study, their taxonomic designation, and their sources are listed in the Appendix A All the bacterial strains had previously been identified as members of *Xanthomonas* spp by fatty acid analysis (Nancy C Hodge, personal communication) The strains of *X campestris* were grown on nutrient agar (Becton Dickson, Cockeysville, MD) Strains of *X albilineans* and *X. fragariae* were grown on Wilbrink s medium (Koike, 1965) Broth cultures were grown 24 hours on a rotatory shaker (150 rpm) at 28°C All strains were streaked on appropriate media and cultures were obtained from single colonies The strains were stored and maintained in tap water at room temperature or in 30% glycerol at -70°C, or both

DNA extraction

Total bacterial genomic DNA was prepared by using the phenol-chloroform extraction procedure described by Ausubel et al (1987) with minor modifications. Bacterial cells were harvested by centrifuging for 2 min at 16,000 g. The pellet was washed once in 1 ml of distilled water, and then resuspended in 567 µl of TE buffer (10 mM Tris Cl, pH 8 0, 1 mM EDTA pH 8 0) Lysis solution containing Proteinase K (Boehringer Mannheim, Indianapolis, IN) and sodium dodecyl sulfate (SDS) (Sigma, St. Louis, MO) were added to final concentrations of 100 µg/ml and 0 5%, respectively The samples were incubated for 1 h at 37°C Sodium chloride and hexadecyltrimethyl ammonium bromide (Sigma) were added for a final concentration of 0 7 M and 1%, respectively and incubated for 10 min at 65°C DNA extraction was performed by sequential chloroform isoamyl alcohol (24 1) and phenol-chloroform isoamyl alcohol (25 24 1) extractions For each extraction, the samples were hand shaken continuously and gently for 10 min and centrifuged for 5 min at 16 000 g After isopropanol precipitation, the DNA pellet was washed with 70% ethanol, and then dried under vacuum for 20 min The pellet was redissolved in 100 µl of TE and stored at 4°C.

DNA amplification

Two sets of oligonucleotide primers from sequences of the *hrp* gene cluster of *Xanthomonas campestris* pv vesicatoria (Bonas et al 1991) were used in this study The primers RST2 and RST3 delineated an 840-bp region and the primers RST21 and RST22 delineated an 1.075-bp region of the complementation groups B and C/D of the *hrp* gene cluster of *X campestris* pv vesicatoria, respectively (Chapter 3, Bonas et al., 1991) Oligonucleotide primers were synthesized with a model 394 DNA Synthesizer (Applied Biosystems, Foster City, CA) by the DNA Synthesis Laboratory University of Florida, Gainesville.

The DNA sequences were amplified in a reaction mixture of 50 µl containing 5 µl of 10X buffer (500 mM KCl, 100 mM Tris-Cl [pH 9 0 at 25°C], 1% Triton X-100) 1.5 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (Boehringer Mannheim), 25 pmol of each primer 1 25 units of *Taq* polymerase (Promega, Madison, WI), and 100 ng of purified template DNA The reaction mixture was overlaid with 50 µl of light mineral oil Thirty amplification cycles were carried out in an automated thermocycler PT-100 60 (MJ Research, Watertown, MA) according to the following profiles 30 sec of denaturation at 95°C, 30 s of annealing at 62°C, and 45 s of extension at 72°C for the primers RST2 and RST3 and 30 s of denaturation at 95°C, 40 s of annealing at 61°C, and 45 s of extension at 72°C for the primers RST21 and RST22. The last extension step for both profiles was extended to 5 min.

The amplified DNA sequences were detected by electrophoresis in 0.9% agarose gels in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.2) according to standard procedures (Ausubel et al., 1987; Sambrook et al., 1989).

Restriction endonuclease analysis

The amplified DNA fragments were restricted with either endonucleases *Cfo*I, *Hae*III, *Sau*3AI, or *Taq*I under conditions specified by the manufacturer (Promega). The restricted fragments were separated by electrophoresis in 4% agarose gels (3% NuSieve and 1% Seakem GTG [FMC BioProducts, Rockland, ME]) in TAE buffer at 8 V/cm. The gel was stained with 0.5 µg of ethidium bromide per ml for 40 min and then destained in 1 mM $MgSO_4$ for 1 hour and photographed over a UV transilluminator (Fotodyne Inc., New Berlin, WI).

Establishment of the restriction fragment patterns

The DNA restriction banding patterns were determined by visual examination of the *hrp*-related fragments digested with each of the four endonucleases. The banding patterns obtained for each fragment were used for comparison with the patterns obtained for homologous fragment amplified from strains of a given pathovar and also from strains of different pathovars of *X. campestris* and of the species *X. fragariae*. A restriction profile number was assigned to each unique DNA restriction banding pattern. This restriction profile number was then used to determine the RFLP group of each strain or group of strains of the plant pathogenic xanthomonads included in this study.

Results

Amplification of the *hrp*-related fragments from strains of *X. campestris* and related *Xanthomonas* spp.

The *hrp*-related fragments delineated by the primers RST2 plus RST3 and primers RST21 plus RST22 were amplified by polymerase chain reaction from plant pathogenic strains representing *X. fragariae* and 28 different pathovars of *X. campestris* (Table 4-1). The fragments amplified were of identical sizes in all cases with both sets of primers (data not shown). Low DNA yield was obtained for the 840-bp *hrp*-related fragment amplified with the primers RST2 and RST3 for all strains of *X. campestris* pv. carotae, *X. campestris* pv. gardneri, *X. campestris* pv. papavericola, *X. campestris* pv. pelargonii, *X. campestris* pv. taraxaci, and two strains of *X. campestris* pv. vitians (Table 4-1). The 840 bp *hrp* fragment was not amplified from a strain of *X. campestris* pv. holcicola (Table 4-1) whereas both *hrp* fragments were not amplified from strains of *X. albilineans*, *X. campestris* pv. celebensis, *X. campestris* pv. secalis, *X. campestris* pv. translucens, and nonpathogenic *X. campestris* (Table 4-1). Moreover, both *hrp* fragments were not amplified also from some strains of a few other pathovars of *X. campestris* which includes *X. campestris* pv. fici, *X. campestris* pv. pelargonii, *X. campestris* pv. phaseoli, *X. campestris* pv. poinsettiicola, and *X. campestris* pv. pruni (Table 4-1).

Establishment of the restriction banding pattern profiles

The *hrp*-related fragments were amplified from 192 strains of plant pathogenic xanthomonads representing *X. fragariae* and 28 different pathovars of *X. campestris* (Table 4-1). Restriction fragment length polymorphism occurred in both *hrp*-related

Table 4-1 Amplification of the *hrp*-related fragments from strains of *Xanthomonas campestris* and related *Xanthomonas* spp

Species/Pathovar	No of strains tested	No of strains with positive amplification of *hrp*-related fragment	
		840 bp	1,075 bp
X. campestris			
pv alfalfae	2	2	2
pv armoraciae	3	3	3
pv begoniae	9	9	9
pv bilvae	1	1	1
pv campestris	9	9	9
pv carotae	7	7(7)^a	7
pv celebensis	1	0	0
pv citri			
canker A	6	6	6
canker B	7	7	7
canker C	6	6	6
pv citrumelo	16	16	16
pv dieffenbachiae	10	10	10
pv fici	8	5	5
pv gardneri	3	3(3)	3
pv glycines	7	7	7
pv holcicola	1	0	1
pv incanae	1	1	1
pv maculifoliigardeniae	1	1	1
pv malvacearum	9	9	9
pv manihotis	1	1	1
pv papavericola	1	1(1)	1
pv pelargonii	8	7(7)	7
pv phaseoli	7	5	5
pv phaseoli "fuscans"	1	1	1
pv physalidicola	1	1	1
pv poinsettiicola	7	4	6
pv pruni	5	4	4
pv raphani	4	4	4
pv secalis	1	0	0
pv taraxaci	1	1(1)	1

Continued on following page

Table 4-1—*Continued*

Species/Pathovar	No of strains tested	No of strains with positive amplification of *hrp*-related fragment	
		840 bp	1,075 bp
pv translucens	1	0	0
pv vesicatoria			
group A	27	27	27
group B	11	11	11
pv vignicola	3	3	3
pv vitians	6	6(2)	6
undetermined and isolated from *Feronia* sp	1	1	1
undetermined and isolated from *Hibiscus* sp	3	1	3
undetermined and isolated from *Strelitzia reginae*	1	1	1
undetermined and nonpathogenic	25	0	0
X albilineans	1	0	0
X fragariae	9	9	9

[a]Number in brackets indicates number of strains which produced weak signal.

fragments amplified from the different plant pathogenic xanthomonads after digestion with frequent-cutting endonucleases *Cfo*I, *Hae*III, *Sau*3AI, and *Taq*I. The combination of *hrp*-related sequences and restriction endonucleases were useful for the establishment of distinct restriction banding patterns for the different groups of plant pathogenic xanthomonads included in this study.

The strains of plant pathogenic xanthomonads were distributed into fifty different groups on the basis of the banding patterns of two *hrp*-related fragments digested with the restriction endonucleases *Cfo*I, *Hae*III, *Sau*3AI, and *Taq*I (Table 4-2). The restriction banding patterns were determined by visual comparisons of the DNA bands separated by electrophoresis of agarose gels (Fig. 4-1 to 4-8). The 1,075-bp *hrp*-related fragment amplified from different strains of xanthomonads and restricted with the endonucleases *Cfo*I and *Hae*III generated the largest number of restriction banding patterns, 23 and 25, respectively (Fig. 4-5 and 4-6). The restriction analysis of the *hrp*-related fragment with these two endonucleases was more discriminatory and was very useful for differentiation of closely related pathovars, such as *X. campestris* pv. armoraciae and *X. campestris* pv. campestris (Table 4-2), or even to distinguish groups of strains within pathovars, such as the case of strains of *X. campestris* pv. dieffenbachiae (Fig. 4-11, Table 4-2) and *X. campestris* pv. citri (Chapter 6, Table 4-2). On the other hand, the restriction analysis with less discriminatory endonucleases, such as *Taq*I, was very helpful to establish the genetic relatedness at pathovar level (Table 4-2). For example, the cruciferous pathogens *X. campestris* pv. armoraciae, *X. campestris* pv. campestris, *X. campestris* pv. incanae, and *X. campestris* pv. raphani all belong to the *Taq*I profile 1 for the 840-bp *hrp*-related fragment and to the *Taq*I profile 3 for the 1,075-bp *hrp*-related fragment (Table 4-2).

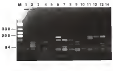


Fig 4-1 Restriction profiles established for the 840-bp *hrp*-related fragment amplified from different strains of plant pathogenic *Xanthomonas* spp and restricted with the endonuclease *Cfo*I Lane M, phage λ restricted with *Pst*I Molecular sizes are given in bases See Table 4-2 for identification of strains of *Xanthomonas* spp for each restriction pattern.

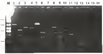


Fig 4-2 Restriction profiles established for the 840 bp *hrp*-related fragment amplified from different strains of plant pathogenic *Xanthomonas* spp and restricted with the endonuclease *Hae*III Lane M phage λ restricted with *Pst*I Molecular sizes are given in bases. See Table 4-2 for identification of strains of *Xanthomonas* spp for each restriction pattern.

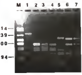


Fig. 4-3 Restriction profiles established for the 840-bp *hrp*-related fragment amplified from different strains of plant pathogenic *Xanthomonas* spp and restricted with the endonuclease *Sau*3AI Lane M, phage λ restricted with *Pst*I Molecular sizes are given in bases See Table 4-2 for identification of strains of *Xanthomonas* spp for each restriction pattern

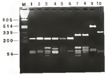


Fig. 4-4 Restriction profiles established for the 840-bp *hrp*-related fragment amplified from different strains of plant pathogenic *Xanthomonas* spp. and restricted with the endonuclease *Taq*I. Lane M, phage λ restricted with *Pst*I. Molecular sizes are given in bases. See Table 4-2 for identification of strains of *Xanthomonas* spp. for each restriction pattern.

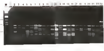


Fig 4-5 Restriction profiles established for the 1,075-bp *hrp*-related fragment amplified from different strains of plant pathogenic *Xanthomonas* spp. and restricted with the endonuclease *Cfo*I Lane M phage λ restricted with *Pst*I Molecular sizes are given in bases See Table 4-2 for identification of strains of *Xanthomonas* spp. for each restriction pattern

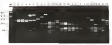


Fig 4-6 Restriction profiles established for the 1 075-bp *hrp*-related fragment amplified from different strains of plant pathogenic *Xanthomonas* spp and restricted with the endonuclease *Hae*III Lane M phage λ restricted with *Pst*I Molecular sizes are given in bases. See Table 4-2 for identification of strains of *Xanthomonas* spp for each restriction pattern

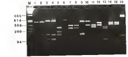


Fig 4-7 Restriction profiles established for the 1,075-bp *hrp*-related fragment amplified from different strains of plant pathogenic *Xanthomonas* spp and restricted with the endonuclease *Sau*3AI Lane M, phage λ restricted with *Pst*I Molecular sizes are given in bases See Table 4-2 for identification of strains of *Xanthomonas* spp for each restriction pattern.

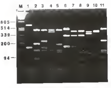


Fig. 4-8. Restriction profiles established for the 1,075 bp *hrp*-related fragment amplified from different strains of plant pathogenic *Xanthomonas* spp. and restricted with the endonuclease *Taq*I. Lane M: phage λ restricted with *Pst*I. Molecular sizes are given in bases. See Table 4-2 for identification of strains of *Xanthomonas* spp. for each restriction pattern.

Table 4-2 Restriction pattern of the 840 and 1,075 bp *hrp*-related DNA fragments amplified from different strains of plant pathogenic *Xanthomonas* spp. and digested with four restriction endonucleases

Species/Pathovar	Strain	840 bp				1,075 bp			
		*Taq*I	*Sau*3AI	*Hae*III	*Cfo*I	*Taq*I	*Sau*3AI	*Hae*III	*Cfo*I
X. campestris									
pv. alfalfae	G 22 (KS)	8[a]	6	16	12	8	11	13	8
	82-1	8	6	16	12	8	13	13	8
pv. armoraciae	63-27, 417, 756	1	3	3	4	3	4	3	2
pv. begoniae	XCB9, X274, X329, X627, X1490, X1492, X1496	1	6	14	10	9	14	20	15
	X610	1	6	14	10	9	14	21	15
	X281	1	6	14	10	9	14	24	22
pv. bilvae	XCB	4	5	9	9	7	10	10	9
pv. campestris	33913, 62-1, 62-9a, 65-6b, 70-3, 70-5, 71-2, 83-1, 83-2	1	3	3	3	3	4	3	2
pv. carotae	#3, #5, #7, #9, #12, #13	nd[b]	nd	nd	nd	3	2	5	2
	#16	nd	nd	nd	nd	3	4	3	2

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Table 4-2—*Continued*

Species/Pathovar	Strain	840 bp				1,075 bp			
		*Taq*I	*Sau*3AI	*Hae*III	*Cfo*I	*Taq*I	*Sau*3AI	*Hae*III	*Cfo*I
pv. citri									
canker A	3213 3340, 9760-2, 9771, T1, 115A	2	5	7	8	7	9	10	7
canker B	B64, B69 B80 B84, B93, B94 B148	4	5	8	9	7	9	11	11
canker C	70C, 338 339, 340, 341,342	4	5	8	9	7	9	11	11
pv. citrumelo	F1 F54, F274, F361, 3166	7	6	16	12	8	11	14	14
	F59, F86	3	6	16	12	8	11	19	8
	F94	3	6	16	12	8	11	15	17
	F306	5	6	16	12	8	11	15	17
	F254, F311	7	6	16	12	8	11	15	8
	F6, F228	8	6	16	14	8	13	15	15
	F100	3	6	16	12	8	12	15	11
	F348	8	6	16	12	8	12	19	16
	F378	3	6	16	14	8	12	14	8

Continued on following page

Table 4-2—*Continued*

Species/Pathovar		Strain	840 bp				1 075 bp			
			*Taq*I	*Sau*3AI	*Hae*III	*Cfo*I	*Taq*I	*Sau*3AI	*Hae*III	*Cfo*I
pv. dieffenbachiae	A	X422, X757, X790, X1272	2	7	12	10	9	15	22	18
		X729, X736, X738, X745	2	7	12	10	9	14	22	18
	B	X260, X763	3	6	13	11	10	14	23	15
pv. fici	A	X125	3	6	16	12	8	11	15	8
		X151	7	6	16	12	8	11	18	11
		X212	5	6	16	12	8	11	18	11
	B	X208, X702	1	4	4	5	5	7	8	5
pv. gardneri		XG101, XV6, 1066	nd	nd	nd	nd	3	3	4	2
pv. glycines	A	G-56, 86-16, 86-17, 86-18, 86-20, 87-2	2	5	6	7	7	9	10	7
	B	1706A	nd	nd	nd	nd	5	7	8	5
pv. holcicola		G-23	nd	nd	nd	nd	11	16	25	23
pv. incanae		9561-1	1	3	3	3	3	4	3	2
pv. maculifoliigardeniae		X22j	2	7	10	10	9	14	24	20

Continued on following page

Table 4-2—*Continued*

Species/Pathovar	Strain	840 bp				1,075 bp			
		*Taq*I	*Sau*3AI	*Hae*III	*Cfo*I	*Taq*I	*Sau*3AI	*Hae*III	*Cfo*I
pv. malvacearum	RIATC, XcmA, XcmB, XcmC, XcmD, XcmE, XcmF, XcmH, XcmN	2	5	6	6	6	9	12	10
pv. manihotis	Xm125D	2	7	11	10	9	14	22	21
pv. papavericola	XP5	nd	nd	nd	nd	3	2	3	3
pv. pelargonii	XCP2, XCP10, XCP17, XCP36, XCP54, XCP58, XCP60	nd	nd	nd	nd	3	2	3	2
pv. phaseoli A	81-19, 82-1, 85-6, XCPH4	2	7	12	10	9	14	22	19
B	82-17	2	5	6	7	7	9	10	7
pv. phaseoli "fuscans"	XP163A	4	5	15	9	7	9	11	12
pv. physalidicola	XP172	3	6	16	12	nd	nd	nd	nd
pv. poinsettiicola B	X87, X202, X259	10	4	5	5	5	8	9	6
A	X352, 071-424	3	6	16	12	8	11	15	8
pv. pruni	X1219L, X1219S, X1220L, X1220S	3	6	16	12	8	11	19	8

Continued on following page

Table 4-2—*Continued*

Species/Pathovar	Strain	840 bp				1075 bp			
		*Taq*I	*Sau*3AI	*Hae*III	*Cfo*I	*Taq*I	*Sau*3AI	*Hae*III	*Cfo*I
pv. raphani	69 2, 69-4B 69-8R	1	3	3	3	4	6	7	4
	16B	1	3	3	4	4	6	7	4
pv. taraxaci	XT11A	nd	nd	nd	nd	3	3	6	2
pv. vesicatoria									
group A	75-3, 82-4 85 10 87-21 87-35T 87-44T 87-48T 89-8 89 10 90-20 90-21, 90-27 90-40 91-66, 91-72 92-11, 92-15 92-16 92 17 92-119, 1712 6107, XV14 XV17	3	6	16	12	8	11	16	17
	92-118, 92-120	3	6	16	13	8	12	17	17
	90-60	3	6	16	12	8	12	17	17
group B	141, 0226A, 0350A 695, 853, 1062 B-3 B-20 BA28-1, BV5-5, XV56	1	1	1	1	1	1	1	1
pv. vignicola	81-30 82-38 G-55	6	7	16	12	7	9	11	11

Continued on following page

Table 4-2—*Continued*

Species/Pathovar		Strain	840 bp				1,075 bp			
			*Taq*I	*Sau*3AI	*Hae*III	*Cfo*I	*Taq*I	*Sau*3AI	*Hae*III	*Cfo*I
pv vitians	A	ICPB101, XVIT, X1215	2	7	12	10	9	14	22	17
	B	XV2	2	5	6	6	6	9	12	10
	C	ICPB164 XV3	nd	nd	nd	nd	3	2	4	2
undetermined and isolated from *Feronia* sp		XCF	4	5	9	9	7	10	10	10
undetermined and isolated from *Hibiscus* sp		X52	nd	nd	nd	nd	5	8	9	6
undetermined and isolated from *Strelitzia reginae*		X198	9	7	16	12	8	11	15	8
X fragariae		GC 6259, GC 6265, X1238, X1241 X1244 X1246, X1292, X1298, X1426	1	2	2	2	2	5	2	13

[a]Restriction pattern group, Number of the restriction pattern groups refer to lanes in Fig 4-1 to 4-8

[b]nd, not determined

Variability of homologous *hrp*-related fragments at species and pathovar levels

Strains of *X. campestris* pv. holcicola, *X. campestris* pv. vesicatoria group B, and *X. fragariae* each produced characteristic restriction patterns for both *hrp*-related fragments digested with four different endonucleases. In the case of the 1,075-bp *hrp*-related fragment amplified from strains of the three taxa and restricted with either *Cfo*I, *Hae*III, *Sau*3AI, or *Taq*I, the restriction banding patterns produced were unique (Table 4-2). Furthermore, the restriction pattern obtained for strains of *X. campestris* pv. vesicatoria group B and *X. fragariae* were identical within each group for all four endonucleases (Table 4-2). In regard to the 840-bp *hrp*-related fragment, the restriction patterns were also unique for the strains of *X. campestris* pv. vesicatoria group B and *X. fragariae* with the exception of the banding patterns obtained by using the restriction endonuclease *Taq*I (Table 4-2). In this case, the restriction pattern of strains of *X. campestris* pv. vesicatoria group B and *X. fragariae* were identical to each other and also to strains of another group of plant pathogenic xanthomonads that included *X. campestris* pv. armoraciae, *X. campestris* pv. begoniae, *X. campestris* pv. campestris, *X. campestris* pv. fici group B, *X. campestris* pv. incanae, and *X. campestris* pv. raphani (Table 4-2). The 840-bp *hrp*-related fragment was not amplified from the strain of *X. campestris* pv. holcicola (Table 4-1).

On the contrary, strains of the other pathovars of *X. campestris* usually produced restriction banding patterns that were common to other groups of xanthomonads in at least one combination of *hrp*-related fragment and restriction endonuclease (Table 4-2). However, the combined profiles of four endonucleases and two *hrp*-related fragments provided differentiation for almost all groups of plant pathogenic xanthomonads included in this study (Table 4-2). Among the exceptions are the strain 9561-1 of *X. campestris* pv. incanae and strain #16 of *X. campestris* pv.

carotae that could not be differentiated from the strains of *X. campestris* pv. campestris (Table 4-2). Other cases include the strain 82.17 of *X. campestris* pv. phaseoli that could not be differentiated from strains of *X. campestris* pv. glycines group A, strains F59 and F86 of *X. campestris* pv. citrumelo were indistinguishable from strains of *X. campestris* pv. pisi, strain X125 of *X. campestris* pv. fici group A was identical to strains of *X. campestris* pv. poinsettiicola group A, strain XV2 of *X. campestris* pv. vitians was identical to strains of *X. campestris* pv. malvacearum and strain X52 isolated from *Hibiscus* sp. was indistinguishable from strains of *X. campestris* pv. poinsettiicola group B (Table 4-2).

The restriction analysis of the *hrp*-related sequences revealed some apparent groupings among the plant pathogenic xanthomonads based on the similarity in the restriction banding patterns of homologous *hrp*-related fragments. The pathogens of cruciferous plants, *X. campestris* pv. armoraciae, *X. campestris* pv. campestris, *X. campestris* pv. incanae, and *X. campestris* pv. raphani have very similar restriction profiles for both *hrp*-related fragments and they formed a very distinct group (Table 4-2). Strains of *X. campestris* pv. armoraciae could be differentiated from *X. campestris* pv. campestris and *X. campestris* pv. incanae only by the restriction analysis of the 840-bp *hrp* related fragment with the endonuclease *Cfo*I (Table 4-2). The restriction patterns obtained for these pathovars were usually identical or very similar with the exception to the restriction patterns of the 1,075-bp fragment for strains of *X. campestris* pv. raphani (Table 4-2). Similarly, the strains of the pathovars *X. campestris* pv. carotae, *X. campestris* pv. gardneri, *X. campestris* pv. papavericola, *X. campestris* pv. pelargonii, *X. campestris* pv. taraxaci, and *X. campestris* pv. vitians group B also formed a unique group, although they cause diseases on different hosts. The restriction analysis of the 1,075-bp *hrp*-related fragment amplified from strains of these pathovars revealed very similar banding patterns (Table 4-2). All strains of the latter pathovars belong to the restriction banding profiles 3 and 2 or 3 of the endonucleases *Taq*I and *Sau*3AI, respectively (Table 4-2). The restriction banding patterns from the strains of these pathovars for the endonucleases *Cfo*I and *Hae*III are also very similar and they have several DNA bands in common (Fig. 4-5 and 4-6). Although the other pathovars of *X. campestris* also showed some trends in regard to the similarity of the restriction patterns for both *hrp*-related fragments, a more discriminating analysis by using some clustering approach may be more appropriate to establish the precise delineation of the groupings.

Variability of the *hrp*-related restriction patterns among strains within different taxa of *Xanthomonas* spp. and pathovars of *X. campestris*

Sequence variation in the *hrp* region was revealed by restriction analysis of both *hrp*-related fragments amplified from strains within the same taxa of plant pathogenic xanthomonads. Further, this variation indicated that these plant pathogens may differ in the genetic variability of the DNA sequence of the *hrp* gene cluster. Whereas some taxa have very uniform restriction patterns for both *hrp*-related sequences, others seem to be comprised of very distinct groups of strains. For instance, the restriction banding patterns for all nine *X. fragariae* strains, originally isolated from diseased material in Florida, California, and Canada (Ann R. Chase, personal communication) were identical to each other when both *hrp*-related fragments were restricted with either *Cfo*I, *Hae*III, *Sau*3AI, or *Taq*I (Fig. 4-9, Table 4-2). Similarly, uniform banding patterns were observed for strains within the pathovars *X. campestris* pv. campestris, *X. campestris* pv. malvacearum, *X. campestris* pv. pelargonii, and *X. campestris* pv. raphani (Table 4-2). Strains within the pathovars *X. campestris* pv. armoraciae, *X. campestris* pv. gardneri, and *X. campestris* pv. vignicola also produced

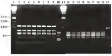


Fig. 4-9 Restriction analysis of the 840-bp *hrp*-related fragments amplified from strains of *Xanthomonas fragariae* and restricted with *Hae*III (Lanes 2 to 10) and *Cfo*I (Lanes 12 to 20) Lanes 1 and 11 phage λ restricted with *Pst*I Lanes 2 and 12, X1238 3 and 13, X1241, 4 and 14 X1244, 5 and 15, X1246, 6 and 16, X1292, 7 and 17, X1298, 8 and 18, X1426 9 and 19 GC6265, 10 and 20, *X. campestris* pv vesicatoria 75-3 Molecular sizes are given in bases.

identical restriction patterns to each other though less than four strains were examined for each of these pathovars (Table 4-2). In the case of the pathovars *X. campestris* pv. gardneri and *X. campestris* pv. pelargonii, only the 1075 bp fragment was examined, because the amplification of the 840-bp *hrp*-related fragment produced very low DNA yield (Table 4-1). Uniform banding patterns were produced by the strains of the pathovars of *X. campestris* pv. carotae, *X. campestris* pv. phaseoli, and *X. campestris* pv. pruni, with the exception of only one single strain within each pathovar (Table 4-2). In another study, nine strains of *X. campestris* pv. begoniae produced identical banding patterns for the restriction analysis of the 840-bp *hrp*-related fragment by using either endonuclease *Cfo*I, *Hae*III, *Sau*3AI, or *Taq*I (Fig. 4-10A). However, the restriction analysis of the 1,075 bp fragment with the endonucleases *Cfo*I or *Hae*III revealed variability in the banding pattern for strains X281 and X610 of *X. campestris* pv. begoniae (Fig. 4-10B). The restriction analysis of this *hrp*-related fragments with the endonucleases *Sau*3AI and *Taq*I revealed that banding patterns were identical for all nine strains of this pathovar (Table 4-2).

In contrast, large variability was found in the restriction analysis of the *hrp*-related fragments amplified from strains of several pathovars of *X. campestris*. This includes the pathovars *X. campestris* pv. citri, *X. campestris* pv. citrumelo, *X. campestris* pv. dieffenbachiae, *X. campestris* pv. fici, *X. campestris* pv. poinsettiicola, *X. campestris* pv. vesicatoria, and *X. campestris* pv. vitians (Fig. 4-11 and 4-12, Table 4-2). Strains of the citrus pathogen *X. campestris* pv. citrumelo were the most heterogeneous and the 17 strains examined were divided into nine different restriction profile groups based on the restriction analysis of the two *hrp*-related sequences (Chapter 6, Table 4-2). On the other hand, strains of the pathovars *X. campestris* pv. citri, *X. campestris* pv. dieffenbachiae, and *X. campestris* pv. vesicatoria produced

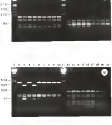


Fig 4-10 Restriction analysis of the (A) 840 bp and (B) 1 075 bp DNA fragments of the *hrp* gene cluster amplified from strains of *Xanthomonas campestris* pv begoniae and restricted with *Hae*III (Lanes 2 to 10) and *Cfo*I (Lanes 12 to 20) Lanes 1 and 11 phage λ restricted with *Pst*I Lanes 2 and 12, X274 3 and 13, X281, 4 and 14, X329; 5 and 15, X610, 6 and 16, X627, 7 and 17, X1490 8 and 18 X1492 9 and 19, X1496, 10 and 20 XCB9 Molecular sizes are given in bases

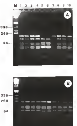


Fig 4-11 Restriction analysis of the (A) 840 bp and (B) 1,075 bp DNA fragments of the *hrp* gene cluster amplified from strains of *Xanthomonas campestris* pv dieffenbachiae and restricted with *Hae*III Lanes M phage λ restricted with *Pst*I Lanes 1, X422, 2, X757, 3, X790, 4 X1272 5 X260, 6, X763, 7, X736, 8, X738, 9, X745, 10, X729 Molecular sizes are given in bases

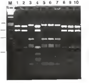


Fig 4-12 Restriction analysis of the 1 075 bp DNA fragments of the *hrp* gene cluster amplified from strains of *Xanthomonas campestris* pv fici (Lanes 1 to 5) and *Xanthomonas campestris* pv poinsettiicola (Lanes 6 to 10) and restricted with *Taq*I Lane M, phage λ restricted with *Pst*I Lanes 1 X125, 2, X151 3 X208 4, X212, 5, X702 6 X87, 7 X202 8 X352 9, 071-424 10 *X campestris* pv vesicatoria 75 3 Molecular sizes are given in bases

distinct restriction patterns that corresponded to the grouping established previously on the basis of pathogenic features (Fig. 4-11, Table 4-2). For instance, the strains of *X. campestris* pv. citri of the citrus canker groups A, B, and C each produced characteristic restriction patterns for all the different combinations of fragment-restriction endonuclease tested (Chapter 6, Table 4-2). Similarly, strains of *X. campestris* pv. vesicatoria groups A and B also produced characteristic restriction banding patterns (Table 4-2). The two groups of *X. campestris* pv. vesicatoria could be differentiated by any combination *hrp* fragment/restriction endonuclease (Table 4-2). Strains of *X. campestris* pv. dieffenbachiae that cause diseases in plants of the family Araceae also produced characteristic restriction patterns (Fig. 4-11, Table 4-2). Among the strains of *X. campestris* pv. dieffenbachiae, three RFLP groups were established on the basis of restriction analysis of the *hrp* related fragments that corresponded to the host of origin. The strains X422, X790, X757, and X1272 were isolated from *Anthurium* sp., strains X260 and X763 were isolated from *Syngonium* sp., and strains X736, X738, X739, and X745 were isolated from *Xanthosoma sigittifolium* (Table 4-2). Further, the strains from *Anthurium* sp. could be distinguished from the *X. sigittifolium* strains only by comparison of the 1 075-bp fragment restricted with the endonuclease *Sau*3AI (Table 4-2).

The strains of *X. campestris* pv. fici and *X. campestris* pv. poinsettiicola each were divided in two different groups based on the distinct restriction banding patterns for both *hrp*-related fragments (Fig. 4-12, Table 4-2). Further, sequence variability was also observed within the groups, particularly for the *X. campestris* pv. fici group A (Table 4-2). However, the banding patterns obtained for the three strains of this group were very similar and they have several bands in common (Fig. 4-12, Table 4-2). The most striking feature of these two pathovars is the similarity of the restriction banding patterns between *X. campestris* pv. fici group A and *X. campestris* pv. poinsettiicola group A, and between *X. campestris* pv. fici group B and *X. campestris* pv. poinsettiicola group B (Fig. 4-12, Table 4-2). Further, the restriction pattern for both fragments amplified from strain X125 of *X. campestris* pv. fici group A were identical to the ones of the strains of *X. campestris* pv. poinsettiicola group A for all combinations of *hrp*-related fragments and restriction endonucleases (Fig. 4-12 and Table 4-2).

Discussion

Several methods have been examined for the differentiation of plant pathogenic xanthomonads with different degrees of success. In the present study, the technique of enzymatic amplification and analysis of specific regions of the bacterial genome was evaluated for the differentiation and identification of plant pathogenic xanthomonads. Unlike other studies where random and less stable regions of the bacterial genome were used (Garde and Bender, 1991, Gilbertson et al., 1989. Hartung, 1992, Hartung et al., 1993, Lazo and Gabriel, 1987. Lazo et al., 1987), I analyzed sequences of the bacterial genome related to the *hrp* gene cluster of *X. campestris* pv. vesicatoria. Although this region of the bacterial chromosome seems to be highly conserved among the plant pathogenic xanthomonads, nonpathogenic xanthomonads lack similarity to the *hrp* genes (Bonas et al., 1991, Stall and Minsavage, 1990). This is certainly a major advantage, because the nonpathogenic xanthomonads is of concern for plant health inspection in certification programs (Gitaitis et al., 1987, 1992). From the results obtained in the present study the DNA amplification of *hrp*-related sequences is highly promising for specific differentiation and identification of a large group of plant pathogenic xanthomonads. DNA sequences related to the *hrp* genes were specifically amplified from bacterial strains representing *X. fragariae* and at least 28 pathovars of *X. campestris*. However, the *hrp*-related sequences were not amplified from strains of the plant pathogenic xanthomonads *X. albilineans*, *X. campestris* pv. celebensis, *X. campestris* pv. secalis, and *X. campestris* pv. translucens, and from a few members of the pathovars *X. campestris* pv. fici, *X. campestris* pv. pelargonii, *X. campestris* pv. phaseoli, *X. campestris* pv. poinsettiicola, and *X. campestris* pv. pruni. Strains of these xanthomonads may not be highly related genetically to the other pathovars of *X. campestris* included in the study as determined by DNA homology studies (Hildebrand et al., 1990, Palleroni et al., 1993, Vauterin et al., 1993) and by the failure to hybridize strongly to the *hrp* clones of *X. campestris* pv. vesicatoria (Chapter 3, Bonas et al., 1991, Stall and Minsavage, 1990). These results support the contention of the presence of differences in the DNA sequences of *Xanthomonas* corresponding to one or both primers used.

Although, no size variation was observed for the *hrp*-related fragments amplified from different plant pathogenic xanthomonads, the restriction analysis of these fragments revealed the presence of sequence variation. Fragment length polymorphisms in the *hrp* regions were further explored for differentiation of the different groups of plant pathogenic xanthomonads. The restriction banding profile established for the two *hrp*-related fragments digested with four endonucleases ranged from 7 to 25 different profiles though only a very few groups of xanthomonads, i.e. *X. campestris* pv. holcicola, *X. campestris* pv. vesicatoria group B, and *X. fragariae*, produced unique restriction banding profiles (Table 4-2). This was somewhat expected because these three groups of xanthomonads seem to be genetically unique (Hildebrand et al., 1990, Palleroni et al., 1993, Vauterin et al., 1993). For example, *X. campestris* pv. vesicatoria group B is genetically and phenotypically very distinct from *X. campestris* pv. vesicatoria group A, although both groups are presently lumped into the same pathovar and cause similar disease symptoms on solanaceous plants (Stall et al., 1994). These two groups of strains have different genetic backgrounds and they are only about 33% similar on the basis of DNA homology (Stall et al., 1994). On the other hand, the majority of the plant pathogenic xanthomonads produced restriction banding patterns that were shared by strains of different groups or pathovars of *X. campestris*. Furthermore, different restriction profiles may have DNA bands in common. The presence of similarities in the restriction banding patterns of strains of different taxa strongly supports the presence of some degree of genetic relatedness among them.

Several pathovars of *X. campestris* were determined to be genetically very closely related to each other on the basis of DNA homology (Hildebrand et al., 1990, Palleroni et al., 1993, Vauterin et al., 1993) and RFLP analysis (Graham et al., 1990; Gottwald et al., 1991, Lazo et al., 1987, Verdier et al., 1993). For example, the pathovars *X. campestris* pv. alfalfae, *X. campestris* pv. begoniae, *X. campestris* pv. cassavae, *X. campestris* pv. citri, *X. campestris* pv. citrumelo, *X. campestris* pv. dieffenbachiae, *X. campestris* pv. glycines, *X. campestris* pv. malvacearum, *X. campestris* pv. manihotis, *X. campestris* pv. phaseoli, *X. campestris* pv. phaseoli "fuscans", *X. campestris* pv. poinsettiicola group A, *X. campestris* pv. vesicatoria group A, and *X. campestris* pv. vignicola belong to the DNA homology group VIII established by Vauterin et al. (1993) whereas the pathovars *X. campestris* pv. armoraciae, *X. campestris* pv. campestris, *X. campestris* pv. incanae, and *X. campestris* pv. raphani belong to the group XII (Vauterin et al., 1993). Similar grouping was obtained in the phylogenetic analysis of the restriction fragment data of the *hrp*-related

DNA sequences (Chapter 5). However, the restriction analysis of the two *hrp* fragments with four restriction endonucleases consistently allowed the differentiation of almost all the pathovars and groups of *X. campestris*, even for those genetically closely related (Table 4-2). In a few cases strains of different groups of plant pathogenic xanthomonads could not be distinguished by restriction analysis of *hrp*-related sequence. These include a strain of *X. campestris* pv. incanae and one of *X. campestris* pv. carotae which were identical to strains of *X. campestris* pv. campestris; a strain of *X. campestris* pv. phaseoli which was identical to strains of *X. campestris* pv. glycines group A, strains F59 and F86 of *X. campestris* pv. citrumelo which were indistinguishable from strains of *X. campestris* pv. pruni, strain X125 of *X. campestris* pv. fici group A which was identical to strains of *X. campestris* pv. poinsettiicola group A, strain XV2 of *X. campestris* pv. vitians which was identical to strains of *X. campestris* pv. malvacearum, and strain X52 isolated from *Hibiscus* sp. was indistinguishable from strains of *X. campestris* pv. poinsettiicola group B.

Strains of a few taxa of the plant pathogenic xanthomonads were homogeneous on the basis of restriction analyses of the *hrp*-related sequences. Strains of *X. fragariae* produced identical and almost unique restriction patterns for all combinations of *hrp*-related fragment and restriction endonuclease. The genetic analysis of the *hrp*-related sequences suggests that strains of *X. fragariae* may be formed by a clonal population even though the strains were isolated from plant materials from different geographic locations. The uniformity of the *X. fragariae* population has also been supported by analysis of fatty acid composition (Yang et al., 1993) and by SDS-PAGE of proteins (Vauterin et al., 1991a). Similarly, strains of some pathovars of *X. campestris* were also highly homogeneous with regard to the restriction banding patterns of the *hrp*-related fragments, i.e. *X. campestris* pv. begoniae, *X. campestris* pv. campestris, *X.*

campestris pv. malvacearum, and *X. campestris* pv. pelargonii. The population structure of *X. campestris* pv. begoniae and *X. campestris* pv. pelargonii has been examined quite extensively by using different methods, such as DNA/DNA hybridization, fatty acid composition and SDS-PAGE of proteins. Both pathovars seem to consist of uniform groups of strains with stable phenotypic features and characteristic fatty acid and protein profiles (Vauterin et al., 1990a, Yang et al., 1993). In the same way, *X. campestris* pv. malvacearum also seems to be a fairly homogeneous group of strains on the basis of SDS-PAGE of proteins and fatty acid analysis (Vauterin et al., 1991a, Yang et al., 1993) despite the fact that this pathovar comprises a large number of physiological races (Brinkerhoff, 1970). Although the restriction analyses of the *hrp*-related fragments also suggest for a clonal population structure in other pathovars of *X. campestris*, i.e. *X. campestris* pv. armoraciae, *X. campestris* pv. gardneri, *X. campestris* pv. pruni and *X. campestris* pv. vignicola, the limited number of strains tested does not allow us to make conclusions with certainty.

In contrast, a striking feature of the different pathovars of *X. campestris* was the high degree of genetic variability in the *hrp*-related sequences for strains within pathovars. For example, strains of *X. campestris* pv. citri were separated in two homogeneous groups on the basis of the restriction endonuclease analysis of the *hrp*-related sequences. One group comprises all strains that corresponds to the citrus canker A of *X. campestris* pv. citri whereas the other group includes strains of the citrus canker B and C forms. Although the citrus canker groups were established on the basis of pathogenic specialization of the strains (Leite, 1990, Stall and Civerolo, 1991) more extensive studies on the genetic and phenotypic characteristics also support this grouping of the strains of *X. campestris* pv. citri (Egel et al., 1991, Gabriel et al., 1988, 1989, Graham et al., 1990, Hartung and Civerolo, 1987). Strains of *X. campestris* pv.

dieffenbachiae also have a high degree of genetic variability. Strains of *X. campestris* pv. dieffenbachiae produced very similar restriction patterns for all combinations of *hrp* fragments-restriction endonucleases and they were divided in three different groups of restriction banding profiles. Further, the strains were accurately identified to their host of origin based on the restriction analysis of the *hrp* related fragments. The genetic heterogeneity of the strains of *X. campestris* pv. dieffenbachiae agrees with the diversity determined on the basis of pathological and physiological features, fatty acid composition, and SDS-PAGE of proteins (Chase et al., 1992, Vauterin et al., 1991a). Moreover, strains of *X. campestris* pv. dieffenbachiae that cause disease on *Syngonium* sp. have been designated *X. campestris* pv. syngonii (Dickey and Zumoff, 1987) although strong support has not been obtained yet to place these strains into a different pathovar (Chase et al., 1992).

In the restriction analysis of the *hrp*-related sequences, the most heterogeneous pathovar was *X. campestris* pv. citrumelo. The seventeen strains of *X. campestris* pv. citrumelo were separated into nine different groups on the basis of the restriction banding profiles (Table 4-2). However, the strains of the highly aggressive group of *X. campestris* pv. citrumelo were homogeneous and comprised a single group (Egel et al., 1991). The high uniformity of the strains of the highly aggressive group of *X. campestris* pv. citrumelo has also been determined in studies of DNA homology, RFLP analyses, fatty acid composition, and SDS-PAGE of proteins (Chapter 3, Egel et al., 1991, Gabriel et al. 1988, 1989; Graham et al., 1990; Hartung and Civerolo, 1987, 1989, Vauterin et al., 1991b). On the other hand, the moderately and weakly aggressive strains of *X. campestris* pv. citrumelo are very diverse, and they are likely to determine the heterogeneity of this pathovar. The diverse nature of the strains of *X.*

campestris pv. citrumelo has also been reported previously (Egel et al., 1991, Gabriel et al., 1988, 1989, Graham et al., 1990, Hartung and Civerolo, 1987, 1989).

The restriction banding profiles of some strains of *X. campestris* pv. citrumelo were very similar or even identical to the profiles of other pathovars of *X. campestris*. For instance the strains F59 and F86 *X. campestris* pv. citrumelo have profiles for both *hrp*-related fragments identical to strains of *X. campestris* pv. pruni whereas other strains of *X. campestris* pv. citrumelo were closely related to strains of *X. campestris* pv. fici A, *X. campestris* pv. poinsettiicola A, and *X. campestris* pv. vesicatoria A (Table 4-2). The close genetic relatedness of strains of *X. campestris* pv. citrumelo to other pathovars of *X. campestris* has also been determined previously (Egel et al., 1991, Gabriel et al., 1988, 1989, Graham et al., 1990, Hartung and Civerolo, 1987, 1989).

The *X. campestris* pathovars, fici, poinsettiicola, vesicatoria, and vitians seem to comprise of a very diverse group of bacteria (Stall et al., 1994, Vauterin et al., 1991a, Yang et al., 1993). Our results on the genetic analysis of the *hrp*-related sequences also corroborate the existence of distinct groups of strains within these pathovars. Further, the grouping obtained based on the analysis of the *hrp*-related sequences agrees very closely with the grouping established previously on the basis of genetic or phenotypic features or both. For example, strains of *X. campestris* pv. vesicatoria were grouped into two distinct groups based on the restriction banding profile of the *hrp*-related sequences (Table 4-2). The groups established based on the *hrp*-related analysis are genetically highly uniform and they correspond to the previous existing groups A and B of *X. campestris* pv. vesicatoria (Stall et al., 1994). On the other hand, the pathovars fici and poinsettiicola have a more complex picture. Strains of both pathovars were also divided into two distinct RFLP groups. Further, the group A of *X. campestris* pv.

fici is genetically closely related to group A of *X. campestris* pv. poinsettiicola whereas the B group of *X. campestris* pv. fici is also genetically closely related to group B of *X. campestris* pv. poinsettiicola. The diversity within the pathovars *X. campestris* pv. fici and *X. campestris* pv. poinsettiicola has also been supported by fatty acid analysis (Nancy C. Hodge, personal communication).

The genetic diversity of strains within pathovars of *X. campestris* is not surprising. The classification of plant pathogenic bacteria at pathovar level was not based initially on the genetics or other intrinsic characteristics of the organism but rather on the host from which the bacteria were isolated (Bradbury, 1984; Dye et al., 1980). Furthermore, comprehensive studies on the genetics and phenotypic characteristics have supported the existence of a high degree of diversity among strains of a given group of plant pathogenic xanthomonads that cause diseases in the same host. Therefore, the plant pathogenic xanthomonads comprise a very complex group of bacteria that might not be easily distinguished. The genetic analysis of the *hrp*-related sequences seems to be a very useful tool for differentiation of pathovars and pathogenic groups of plant pathogenic xanthomonads. Furthermore, the diversity or uniformity of the different taxa of xanthomonads assessed on the basis of restriction analysis of *hrp*-related sequences apparently agrees very closely with the existing groups established by using other methods. However, the restriction banding profiles generated for the *hrp*-related fragments may be an easier and more discriminating approach for identification of plant pathogenic xanthomonads, compared to other methods such as genomic fingerprinting or RFLP analysis by using random or specific DNA probes. DNA fingerprinting by digestion of the entire bacterial genome with restriction endonuclease usually produces very complex patterns that are difficult to interpret (Graham and Cooksey, 1989; Hartung and Civerolo, 1987; Vauterin et al., 1993).

whereas RFLP analysis using DNA probes requires hybridization techniques. More extensive work is necessary, however, to characterize the *hrp* sequence variation in other groups of plant pathogenic xanthomonads. Furthermore DNA-DNA hybridization data is also necessary to determine the consistency of the grouping established based on such a small region of the bacterial genome as the *hrp* gene cluster. The restriction data may also be useful to establish the genetic evolutionary relationship of the *hrp* genes among the different plant pathogenic xanthomonads.

CHAPTER 5

PHYLOGENETIC ANALYSIS PLANT PATHOGENIC *Xanthomonas* BASED ON DNA SEQUENCES RELATED TO THE *hrp* GENES

The genus *Xanthomonas* Dowson 1939 includes bacteria that occur worldwide and cause economically important diseases on many plants. The host range of the xanthomonads spans over 392 mono and dicotyledonous plant species (Hayward 1993 Leyns et al., 1984) *X campestris* is certainly the most complex species among the xanthomonads This species is highly diverse and comprises at least 125 different pathovars (Bradbury 1984 Dye et al 1980 Hayward, 1993) The classification of the plant pathogenic xanthomonads has been based largely on the capability of the bacterial strain to cause a characteristic disease (Bradbury 1984 Dye et al., 1980, Vauterin et al , 1990a, Young et al., 1992) Consequently, bacteria with features different from pathogenicity may be classified under the same taxonomic unit. On the other hand, genetically similar bacteria may be placed in different taxa because they cause diseases on different plants

The establishment of relationships among the different pathovars and species of *Xanthomonas* has been attempted by using different approaches such as metabolic fatty acid, and protein profiling (Chase et al , 1992, Hildebrand et al , 1993, Hodge et al., 1992, Van den Mooter and Swings, 1990, Vauterin et al , 1991b) and nucleic acid analyses (Hildebrand et al., 1990 Palleroni et al., 1993 Vauterin et al., 1993) Although the pathovars of *X campestris* have almost identical biochemical and physiological characteristics (Van den Mooter and Swings, 1990) diversity seems to exist among the strains of *X campestris* as demonstrated by protein and fatty acid profiles (Vauterin et al , 1991a Yang et al., 1993) Further genetic analyses of the xanthomonads by DNA-DNA hybridization has revealed a diverse genetic background (Hildebrand et al., 1990 Palleroni et al 1993 Vauterin et al 1992)

A characteristic feature of plant pathogenic xanthomonads is the presence of a genomic region that contains genes (*hrp*) for the hypersensitive and pathogenic reactions on plants This *hrp* gene cluster is required for plant pathogens to cause disease on susceptible hosts and hypersensitive reaction (HR) on resistant, or on nonhost plants (Willis et al., 1991) Besides the xanthomonads, the *hrp* genes have also been found in other Gram negative plant pathogenic bacteria of the genera *Erwinia* (Beer et al 1991) and *Pseudomonas* (Boucher et al , 1987 Huang et al , 1990 Lindgren et al 1986) Furthermore, the *hrp* genes seem to be highly conserved among different bacteria at the structural and functional levels (Fenselau et al 1992, Gough et al , 1992, Hwang et al 1992) The *hrp* genes of plant pathogenic bacteria are also similar at the protein level to virulence determinants of bacteria of the genus *Yersinia*, pathogens of animals (Fenselau et al , 1992, Gough et al , 1992) On the other hand, nonpathogenic bacteria that are unable to cause disease or HR on plants lack DNA similar to the *hrp* genes sequence (Chapter 3, Bonas et al , 1991, Lindgren et al , 1986, Stall and Minsavage, 1990)

Conservation of the *hrp* genes at the structural and functional level among different plant pathogenic xanthomonads is well documented. The *hrp* genes are organized in a large cluster in the bacterial genome (Arlat et al 1991 Bonas et al , 1991) although an additional small *hrp* region unrelated to the major *hrp* genes has also been identified (Kamoun and Kado 1990 Kamoun et al 1992). Southern hybridization analyses using the *hrp* genes of *Xanthomonas campestris* pv vesicatoria as probes revealed the presence of sequence similarity in the genomic DNA of several pathovars of *X. campestris* and related species of *Xanthomonas* (Chapter 3, Bonas et al., 1991; Stall and Minsavage, 1990). Further, the *hrp* genes of the xanthomonads seem to hybridize to genomic DNA of *P. solanacearum* when low stringency conditions are used (Arlat et al., 1991; Boucher et al., 1987). The *hrp* gene clusters of the xanthomonads are also functionally interchangeable. Heterologous complementation of the *hrp* genes has been achieved for several pathovars of *X. campestris* (Chapter 3; Arlat et al., 1991; Bonas et al., 1991). Despite all the information accumulated regarding the *hrp* genes, little is known about the evolutionary relationship among the *hrp* genes of the xanthomonads.

Inferences of the relationships of *hrp* genes of the plant pathogenic xanthomonads based on molecular phylogeny would certainly improve the understanding of the genetics of pathogenicity of these plant pathogens. Dissimilar *hrp* gene clusters in strains with relatively divergent genetic background might indicate that the *hrp* region has coevolved with the rest of the genome from a common bacterial ancestor. On the contrary, similar *hrp* gene clusters in strains with relatively divergent genetic background might indicate a more recent horizontal genetic movement between bacterial strains. Alternatively, variability in the genetic relatedness between bacterial strains for different regions of the *hrp* genes may support the hypothesis of different origins of the *hrp* genes. In this study, I examined the evolutionary relationship of two DNA sequences of the *hrp* genes from different plant pathogenic xanthomonads. DNA fragments of the bacterial genome related to the *hrpB* and *hrpC/D* complementation groups of the *hrp* gene cluster of *X. campestris pv.* vesicatoria (Chapter 3, Bonas et al., 1991) were enzymatically amplified from different plant pathogenic xanthomonads and then digested with frequent cutting restriction endonucleases. The restriction fragment data were used to establish the genetic relationship of the *hrp* genes between strains and to infer the phylogeny of this region of the bacterial genome for different plant pathogenic xanthomonads.

Materials and Methods

Bacterial strains and culture conditions

The bacterial strains used in this study and their sources are listed in appendix A. The identity of the bacterial strains used here was confirmed by fatty acid analysis (N.C. Hodge, personal communication). Strains of *X. campestris* were grown on nutrient agar (Becton Dickinson, Cockeysville, MD) at 28°C, with the exception of strains of *X. campestris* pv. citri group B that were grown on a sucrose based medium (Canteros de Echenique et al., 1985). Strains of *X. fragariae* were cultivated on Wilbrink's medium (Koike, 1965).

DNA manipulations

Total genomic DNA of each strain was isolated by phenol-chloroform extraction and ethanol precipitation essentially as described by Ausubel et al. (1987). The restriction endonuclease analyses of the DNA fragments amplified from different bacterial strains were accomplished by using the frequently cutting endonucleases *Cfo*I, *Hae*III, *Sau*3AI, and *Taq*I under the conditions specified by the manufacturer (Promega, Madison, WI). The restricted fragments were resolved by electrophoresis in 4% agarose gels (3% NuSieve and 1% SeaKem GTG [FMC BioProducts, Rockland, ME]) in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.2) at 8 V/cm.

DNA amplification

Two sets of oligonucleotide primers selected from the nucleotide sequence of the *hrp* gene cluster of *X. campestris* pv. vesicatoria were used in this study. Primers RST2 plus RST3 delineated an 840-bp fragment and RST21 plus RST22 delineated a 1,075-bp fragment of the complementation groups *hrp*B and *hrp*C/D of *X. campestris* pv. vesicatoria, respectively (Chapter 3). The primers were used in polymerase chain reaction for specific amplification of homologous *hrp*-related DNA fragments from different plant pathogenic xanthomonads. The reaction conditions and polymerase chain reaction cycles were previously described (Chapter 3).

Data analysis

The electrophoretic patterns of the *hrp*-related DNA fragments restricted with each of the four endonucleases were used to determine the restriction banding profile for each bacterial strain. The codes 0 and 1 were assigned according to the absence or presence of each DNA band, respectively. The genetic relationship between strains was estimated based on the resulting matrix by determining the proportion of shared DNA fragments (F). The equation proposed by Nei and Li (1979), $F = 2n_{xy} / (n_x + n_y)$, where n_{xy} is the number of fragments shared by both strains, and n_x and n_y are the total number of fragments for each strain, was used to estimate the proportion of shared fragments (F) by using a computer program (Appendix B) written for the SAS system (SAS Institute Inc., Cary, NC). The genetic divergence between strains was determined as the estimate of the number of nucleotide substitutions per site (δ), on the basis of the proportion of shared DNA fragments (Nei and Li, 1979). The number of nucleotide substitutions per site (δ) was calculated based on the iterative method suggested by Nei and Li (1979). A program written for the SAS system was used for the calculations (Appendix C).

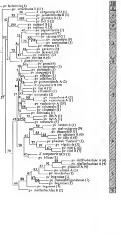
The evolutionary relationship among strains was examined by using programs from the computer package PHYLIP (Felsenstein, 1991, 1993). Phylogenetic trees were inferred for each of the two *hrp* regions individually by using a parsimony criterion and a distance matrix method. The restriction fragment data encoded 0 or 1 were used as input for reconstruction of an unrooted phylogenetic tree by the Wagner parsimony criterion of the BOOT program (Felsenstein, 1991). The strains G-23 of *X. campestris* pv. holcicola and XV56 of *X. campestris* pv. vesicatoria group B were taken as the outgroups to infer the topology of the phylogenetic trees for the *hrpC/D* and *hrpB* regions, respectively. No assumptions were made for the ancestral character state and the confidence intervals of the estimates of the inferred phylogenetic trees were determined by analyzing a total of 100 bootstrap samples (Felsenstein, 1985, 1991). The distance matrix method unweight pair-group method with arithmetic mean (UPGMA) of phylogenetic reconstruction (Nei, 1987) was also applied to the data for reconstruction of a rooted phylogenetic tree. The option UPGMA of the program NEIGHBOR (Felsenstein, 1993) was performed for this analysis. The input data consisted of the estimates of the number of nucleotide substitutions per site (δ) between strains determined by the method developed by Nei and Li (1979). For both methods, the data of each strain were entered in a random order.

Results

DNA fragments related to the *hrpB* and *hrpC/D* complementation groups of the *hrp* genes of *X. campestris* pv. vesicatoria were amplified from strains representing the species *X. fragariae* and 29 pathovars of *X campestris* included in the study (data not shown) However, the amplification of the *hrpB*-related fragment from *X campestris* pv carotae, *X campestris* pv gardneri *X campestris* pv glycines B, *X campestris* pv papavericola, *X campestris* pv pelargonii, *X. campestris* pv vitians C, and a strain of *X. campestris* from *Hibiscus* sp usually produced low yield of DNA whereas no amplification of this fragment was obtained from *X campestris* pv holcicola These strains were not included in the phylogenetic analysis of the *hrpB* region The *hrp*-related fragments amplified with each set of oligonucleotide primers from different plant pathogenic xanthomonads were of identical size

Unrooted parsimony analyses were carried out on 100 and 63 restriction fragment data sets produced by the digestion of the *hrpC/D*- and *hrpB*-related fragments, respectively with four different frequent-cutting restriction endonucleases The phylogenetic trees inferred for each amplified fragment are very similar in topology to each other (Fig 5-1 and 5-2) Ten major clades were identified in the analysis of the *hrpC/D*-related fragment amplified from the plant pathogenic xanthomonads (Fig 5-1) Clades 1, 2, 4, 9, and 10 include only a single taxon whereas the remaining clades are comprised by taxa representing different pathovars of *X campestris* (Fig 5-1 and 5-2). Further the members of each clade can be easily identified based on the restriction banding profile of the *hrpC/D*-related fragment produced with the endonuclease *Taq*I (Table 5 1) The result of the phylogenetic analysis of the *hrpB* region is consistent with the phylogeny determined for the *hrpC/D* region with a few important differences The clades 3 and 4 which contain pathovars of *X. campestris* that cause diseases on brassica plants merged in a larger single clade whereas the single member of clade 9, *X campestris pv* dieffenbachiae B were placed in clade 7 (Fig. 5-2) Another difference is the shift of the strains of *X campestris pv*

Fig. 5-1 Unrooted phylogenetic tree inferred from restriction analysis data of the 1,075 bp DNA fragment related to the *hrpC/D* complementation group of the *hrp* genes of *Xanthomonas campestris* pv. vesicatoria generated by the Wagner parsimony criterion. The values on each node indicate the levels of support derived from 100 bootstrapped trees. The shaded boxes delineate the major clades identified in the analysis of the *hrpC/D*-related DNA fragment. Also included are the number of strains examined for each taxa (numbers in parenthesis).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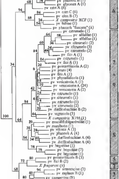
pv poinsettiicola B (3)
pv glycines B (1)
pv ilicі B (2)
pv raphani B (1)
pv raphani A (3)
pv papavericola (1)
pv pelargonii (7)
pv carotae B (1)
pv campestris (9)
pv armoraciae (3)
pv incanae (1)
pv gardneri (3)
pv juglandis (1)
pv vitians C (2)
pv carotae A (6)
X fragariae (9)
pv pruni (4)
pv citrumelo (7)
pv citrumelo (1)
pv citrumelo (1)
pv alfalfae (1)
pv alfalfae (1)
pv poinsettiicola A (2)
X campestris X198
pv fici A (1)
pv citrumelo (2)
pv citrumelo (2)
pv vesicatoria A (2)
pv vesicatoria A (1)
pv vesicatoria A (24)
pv citrumelo (1)
pv citrumelo (1)
pv citrumelo (1)
pv fici A (1)
pv fici A (1)
pv citrumelo (5)
pv vitians B (1)
pv malvacearum (9)
pv phaseoli B (1)
pv glycines A (6)
pv citri A (6)
pv phaseoli "fuscans" (1)
pv vignicola (3)
pv citri C (6)
pv citri B (7)
X campestris XCF (1)
pv bilvae (1)
pv dieffenbachiae A (4)
pv dieffenbachiae A (4)
pv phaseoli A (4)
pv vitians A (3)
pv manihotis (1)
pv begoniae (1)
pv ranunculi/fragariae (1)
pv begoniae (1)
pv begoniae (7)
pv dieffenbachiae B (2)
100
93
96
100
100
42
47
22
33
54
20
73
31
43
73
35
27
63
28
5
47
3
13
33
16
21
17
8
93
37
18
66
6
92
43
21
43
99
40
88
68
61
77
97
67
78
45
37
79
98
91
57
83
47
47
38
88
66
10
1
2
5
3
4
7
6
8
9

Fig. 5.2. Unrooted phylogenetic tree inferred from restriction analysis data of the 840-bp DNA fragment related to the *hrpB* complementation group of the *hrp* genes of *Xanthomonas campestris* pv. vesicatoria generated by the Wagner parsimony criterion. The values on each node indicate the levels of support derived from 100 bootstrapped trees. The shaded boxes delineate the major clades identified in the analysis of the *hrpC/D*-related DNA fragment. Also included are the number of strains examined for each taxa (numbers in parenthesis).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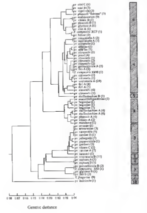
pv malvacearum (9)
pv vitians B (1)
pv phaseoli B (1)
pv glycines A (6)
pv citri A (6)
pv citri C (6)
pv citri B (7)
X campestris XCF (1)
pv bilvae (1)
pv phaseoli "fuscans" (4)
pv citrumelo (1)
pv alfalfae (1)
pv alfalfae (1)
pv citrumelo (2)
pv citrumelo (5)
pv citrumelo (2)
pv fici A (1)
pv citrumelo (1)
pv fici A (1)
pv poinsettiicola A (2)
pv pruni (4)
pv fici A (1)
pv physalidicola (1)
pv vesicatoria A (1)
pv vesicatoria A (24)
pv vesicatoria A (2)
pv citrumelo (1)
pv citrumelo (1)
pv citrumelo (1)
pv citrumelo (2)
pv dieffenbachiae B (2)
pv vignicola (3)
X campestris X198 (1)
pv maculifoliigardeniae (1)
pv manihotis (1)
pv vitians A (3)
pv phaseoli A (4)
pv dieffenbachiae A (4)
pv dieffenbachiae A (4)
pv begoniae (1)
pv begoniae (7)
pv begoniae (1)
pv poinsettiicola B (3)
pv fici B (2)
X fragariae (9)
pv armoraciae (3)
pv raphani B (1)
pv campestris (9)
pv lucanae (1)
pv raphani A (3)
84
42
74
68
94
84
88
26
54
64
58
60
36
14
34
44
86
20
8
4
18
10
42
24
4
14
20
84
44
34
100
64
20
30
28
54
34
60
78
78
80
72
68
94
40
80
32
1
6
7
9
8
5
2
3
4

vignicola from clade 6 to clade 7 in the phylogenetic analyses of the *hrpC/D* and *hrpB* regions, respectively (Fig. 5-1 and 5-2).

The results of the bootstrapped resampling analyses presented in the branches of the trees largely support the monophyletic nature of the major clades in almost all the cases (Fig. 5-1 and 5-2). Further, the topology of these trees reconstructed by the parsimony procedure is consistent with the phylogenetic analyses based on the distance matrix approaches UPGMA (Fig. 5-3 and 5-4) and neighbor-joining (not shown). Further, the UPGMA analyses also indicate that the strains G-23 of *X. campestris* pv. holcicola and XV56 of *X. campestris* pv. vesicatoria group B taken to infer the topology of the unrooted parsimony trees for the *hrpC/D* and *hrpB* regions, respectively, were appropriate outgroups because they were basal to the remainder of *Xanthomonas* spp. included in this study (Fig. 5-3 and 5-4).

The present phylogenetic framework supports the monophyletic nature of the *hrp* gene sequences of plant pathogenic xanthomonads that belongs to different pathovars of *X. campestris* and cause diseases in different hosts. For instance, clade 6 comprises the pathovars *X. campestris* pv. citri, *X. campestris* pv. malvae, *X. campestris* pv. glycines A, *X. campestris* pv. malvacearum, *X. campestris* pv. phaseoli B, *X. campestris* pv. phaseoli "fuscans" and *X. campestris* pv. vitians B (Fig. 5-1 and 5-2). Although these pathogens cause diseases on different plants, there is strong agreement in the parsimony analyses to indicate a common bacterial ancestor for both *hrp* genes regions of these pathovars. The bootstrap resampling analyses support the branching of this clade at 78% and 94% for the *hrpC/D* and *hrpB* regions, respectively (Fig. 5-1 and 5-2). The estimates of the similarity between *hrp* genes for each region also indicates a close genetic relatedness for the members within this clade. The estimates of the similarity of the *hrp* genes region within clade 6 ranged from 0.65 to 1.00 and from

Fig 5 3 Rooted phylogenetic tree inferred from restriction analysis data of the 1 075-bp DNA fragment related to the *hrpC/D* complementation group of the *hrp* genes of *Xanthomonas campestris* pv vesicatoria generated by the method unweight pair-group method with arithmetic mean (UPGMA) The shaded boxes delineate the major clades identified in the analysis of the *hrpC/D*-related DNA fragment Also included are the number of strains examined for each taxa (numbers in parenthesis)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pv citri B (7)
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pv malvacearum (9)
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pv phaseoli B (1)
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pv alfalfae (1)
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pv citrumelo (2)
pv poinsettiicola A (2)
pv fici A (1)
X. campestris X498 (1)
pv citrumelo (1)
pv citrumelo (1)
pv vesicatoria A (24)
pv fici A (1)
pv fici A (1)
pv citrumelo (6)
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pv dieffenbachiae B (2)
pv maculifoliigardeniae (1)
pv begoniae (1)
pv begoniae (1)
pv begoniae (7)
pv dieffenbachiae A (4)
pv dieffenbachiae A (4)
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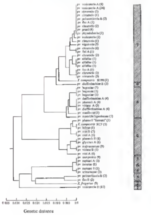
pv vitians A (3)
pv manihotis (1)
pv incanae (1)
pv armoraciae (3)
pv campestris (9)
pv carotae B (1)
pv pelargonii (7)
pv papavericola (1)
pv gardneri (3)
pv vitians C (2)
pv carotae A (7)
pv taraxaci (1)
pv vesicatoria B (11)
pv raphani A (3)
pv raphani B (1)
pv poinsettiicola B (3)
X. campestris X52 (1)
pv glycines B (1)
pv fici B (2)
X. fragariae (9)
pv holcicola (1)
6
9
8
3
4
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Genetic distance

Fig 5-4 Rooted phylogenetic tree inferred from restriction analysis data of the 840-bp DNA fragment related to the *hrpB* complementation group of the *hrp* genes of *Xanthomonas campestris* pv vesicatoria generated by the method unweight pair-group method with arithmetic mean (UPGMA) The shaded boxes delineate the major clades identified in the analysis of the *hrpC/D*-related DNA fragment Also included are the number of strains examined for each taxa (numbers in parenthesis)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pv fici A (1)
pv citrumelo (2)
pv pruni (4)
pv physalidicola (1)
pv vesicatoria (2)
pv citrumelo (1)
pv vignicola (3)
pv citrumelo (4)
pv fici A (1)
pv citrumelo (2)
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pv alfalfae (1)
pv alfalfae (1)
pv fici A (1)
pv citrumelo (2)
pv citrumelo (5)
X. campestris X198 (1)
pv dieffenbachiae B (2)
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pv begoniae (1)
pv dieffenbachiae A (4)
pv phaseoli A (4)
pv vitians A (5)
pv dieffenbachiae A (6)
pv manihotis (1)
pv mangiferaeindicae (1)
pv phaseoli "fuscans" (1)
X. campestris XCF (1)
pv lalvae (1)
pv citri B (7)
pv citri A (6)
pv phaseoli B (1)
pv glycines A (6)
pv malvacearum (9)
pv vitians B (1)
pv citri A (6)
pv campestris (9)
pv raphani A (3)
pv incanae (1)
pv raphani B (1)
pv armoraciae (3)
pv poinsettiicola B (3)
pv fici B (2)
X. fragariae (9)
pv vesicatoria B (13)
7
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.0
Genetic distance

0.53 to 1.00 for the *hrpC/D* and *hrpB* regions, respectively (Table 5-1). Similarly, the clades 3, 5, 7, and 8 also contain pathovars of *X. campestris* that cause diseases on different plants but are genetically very closely related regarding the *hrp* genes for both regions examined (Table 5-1). The bootstrap resampling analyses further substantiate the monophyletic nature of the gene cluster for these clades (Fig. 5-1 and 5-2).

On the contrary, the inferred phylogenetic analysis also indicates that strains which are classified under the same pathovar, because they cause similar diseases on the same host plants, may have very distinct *hrp* sequences. *X. campestris* pv. vesicatoria that causes disease on solanaceous plants is known to contain at least two diverse groups of strains (Stall et al., 1994). The two groups of this pathovar also show distinct *hrp* gene sequences (Fig. 5-1 and 5-2). Strains of *X. campestris* pv. vesicatoria B comprise the sole taxon of the clade 1 whereas the strains of *X. campestris* pv. vesicatoria A belong to the clade 7 (Fig. 5-1 and 5-2). The clade 7 is the largest one and includes several pathovars of *X. campestris* (Fig. 5-1 and 5-2). The placement of the two groups of *X. campestris* pv. vesicatoria in two distant and distinct clades are further supported by the bootstrap resampling analysis (Fig. 5-1 and 5-2). The estimated similarity between the two groups of *X. campestris* pv. vesicatoria in relation to the *hrp* regions ranged from 0.33 to 0.38 and from 0.50 to 0.51 for the *hrpC/D* and *hrpB* regions, respectively. In addition, the three subgroups identified in *X. campestris* pv. vesicatoria A are monophyletic and genetically closely related for both *hrp* gene regions (Fig. 5-1 and 5-2). Several other pathovars of *X. campestris* accommodate groups of strains with very diverse *hrp* genes, i.e. *X. campestris* pv. fici, *X. campestris* pv. glycines, *X. campestris* pv. phaseoli, *X. campestris* pv. poinsettiicola, and *X. campestris* pv. vitians (Fig. 5-1 and 5-2, Table 5-2).

Table 5.1 Within clade similarity values generated by comparison of the restriction profiles obtained by digestion of the *hrp*-related fragments amplified from plant pathogenic *Xanthomonas* spp. with restriction endonuclease enzymes.

Clade[a]	*Taq*I group[b]	Number of taxa in the clade[c]		Similarity within clade	
		hrpB	*hrpC/D*	*hrpB*	*hrpC/D*
1	1	1	1	–	–
2	2	1	1	–	–
3	3	}5	10	0.96-1.00[d,e]	0.78-1.0
4	5		2	0.96	1.00
5	5	2	4	0.87[f]	0.77-1.00
6	6 and 7	10	11	0.53-1.00	0.65-1.00
7	8	22	20	0.77-0.78	0.67-1.00
8	9	9	9	0.84-1.00	0.64-0.96
9	10	1	1	–	–
10	11	na[g]	1	–	–

[a]Clade established on the basis of the phylogenetic analysis of the restriction fragment data of the 1 075-bp fragment related to the *hrpC/D* region of the *hrp* genes of *X. campestris* pv. vesicatoria.

[b]Group established based on the restriction profile of the 1 075-bp fragment related to the *hrpC/D* region of the *hrp* genes of *X. campestris* pv. vesicatoria restricted with the endonuclease *Taq*I (see Chapter 4)

[c]Number of taxa determined based on the phylogenetic analysis of each *hrp* region individually

[d]Values are the similarities estimated by using the equation proposed by Nei and Li (1979) for each *hrp*-related fragment restricted with either endonuclease *Cfo*I, *Hae*III, *Sau*3AI, and *Taq*I

[e]Only three taxa were compared.

[f]Only two taxa were compared.

[g] na, not applicable.

Table 5-2. Similarity values between clades of plant pathogenic *Xanthomonas* spp generated based on the restriction profiles of the DNA fragments related to the *hrpB* and *hrpC/D* complementation groups of the *hrp* genes of *X campestris* pv vesicatoria

	Clade[a]									
	1	2	3	4	5	6	7	8	9	10
1	--	0.25[b]	0.50-0.62	0.41	0.32-0.42	0.42-0.50	0.33-0.46	0.25-0.44	0.46	0.38
2	0.40	--	0.36-0.46	0.32	0.33-0.36	0.25-0.33	0.17-0.25	0.08-0.22	0.14	0.21
3	0.54-0.60	0.52-0.57	--	0.41-0.50	0.39-0.51	0.24-0.46	0.23-0.40	0.18-0.47	0.25-0.40	0.27-0.32
4	0.54-0.50	0.52-0.57	0.96-1.00	--	0.40-0.51	0.29-0.46	0.27-0.41	0.27-0.43	0.40	0.23
5	0.43-0.53	0.62-0.63	0.55-0.60	0.55-0.68	--	0.23-0.37	0.23-0.37	0.14-0.34	0.20-0.26	0.18-0.23
6	0.47-0.56	0.37-0.48	0.51-0.58	0.51-0.58	0.34-0.52	--	0.40-0.74	0.18-0.44	0.32-0.58	0.13-0.18
7	0.38-0.51	0.24-0.41	0.41-0.57	0.41-0.57	0.39-0.56	0.41-0.94	--	0.36-0.67	0.49-0.74	0.13-0.21
8	0.53-0.59	0.50-0.56	0.49-0.58	0.49-0.59	0.34-0.62	0.34-0.79	0.59-0.77	--	0.58-0.75	0.23
9	0.52	0.28	0.53-0.58	0.53-0.58	0.34-0.43	0.48-0.78	0.65-0.85	0.25-0.62	--	0.13-0.22
10	nd[c]	nd	nd	nd	nd	nd	nd	nd	nd	--

[a] Clade established based on the restriction profile on the basis of the phylogenetic analysis of the restriction fragment data of the 1 075 bp fragment related to the *hrpC/D* region of the *hrp* genes of *X campestris* pv vesicatoria, restricted with the endonuclease *Taq*I

[b] Values are the similarities estimates by using the equation proposed by Nei and Li (1979) for the 1 075 bp DNA fragment related to the *hrpC/D* upper triangular matrix and for the 840 bp DNA fragment related to the *hrpB* lower triangular matrix, restricted with either endonuclease *Cfo*I *Hae*III, *Sau*3A1 and *Taq*I

[c] nd, not determined

Another evolutionary relationship revealed by the phylogenetic analysis of the *hrp* genes of the xanthomonads is the variability in the relatedness between strains depending on the *hrp* region examined. For example, *X. campestris* pv. vignicola clustered in clade 6 in the phylogenetic analysis for the *hrpC/D* region (Fig. 5-1), and the value of 78% for the bootstrap resampling supports the monophyletic nature of the *hrp* region for this clade (Fig. 5-1). Further, similarity indices for the different pathovars within this clade ranged from 0.67 to 1.00 (Tables 5-1). In comparison, the similarity of *X. campestris* pv. vignicola to the members of clade 7 ranged from 0.42 to 0.69 (Table 5-2 and 5-3). On the other hand, the strains *X. campestris* pv. vignicola were placed into clade 7 in the phylogenetic analysis of the *hrpB* region (Fig. 5-2), and this was strongly supported by the bootstrap resampling with a value of 84% of the bootstrapped trees (Fig. 5-2). The genetic relatedness of *X. campestris* pv. vignicola to the other members of the clade 7 ranged from 0.76 to 0.94 for the *hrpB* region whereas the similarity of this region between *X. campestris* pv. vignicola strains and the members of the clade 6 ranged from 0.53 to 0.61 (Table 5-3).

Strains of *X. campestris* pv. raphani also comprise an unique case. The phylogenetic analysis of the *hrpC/D*-related region of *X. campestris* pv. raphani revealed that the two closely related groups were placed in clade 4 (Fig. 5-1). The bootstrap resampling analysis also supports 100% the monophyletic nature of this clade (Fig. 5-1). The genetic similarity of the strains in clade 4 to strains of clade 3 which contains the other brassica pathogens i.e. *X. campestris* pv. armoraciae, *X. campestris* pv. campestris, and *X. campestris* pv. incanae ranged from 0.41 to 0.50 (Table 5-2). In contrast, the analysis of the *hrpB* region showed that the *hrp* region for the strains of *X. campestris* pv. raphani and strains of the other three pathovars of *X. campestris* are

Table 5-3 Similarity values between *Xanthomonas campestris* pv. vignicola and selected members of the clades 6 and 7 generated by comparison of the endonuclease profiles of the DNA fragments related to the *hrpB* and *hrpC/D* complementation groups of the *hrp* genes of *X. campestris* pv. vesicatoria.

	Similarity of the *hrp*-related sequences of *X. campestris* pv. vignicola	
	hrpB	*hrpC/D*
Clade 6[a]		
X. campestris		
pv. bilvae	0.61[b]	0.71
pv. citri A	0.53	0.75
pv. glycines A	0.58	0.75
pv. malvacearum	0.59	0.67
pv. phaseoli "fuscans"	0.58	0.83
pv. vitians B	0.59	0.67
Clade 7		
X. campestris		
pv. alfalfae	0.80	0.50
pv. citrumelo	0.87	0.53
pv. fici A	0.94	0.46
pv. poinsettiicola A	0.94	0.43
pv. pruni	0.94	0.46
pv. vesicatoria A	0.94	0.46

[a] Clade established on the basis of the phylogenetic analysis of the restriction fragment data of the 1,075-bp fragment related to the *hrpC/D* region of the *hrp* genes of *X. campestris* pv. vesicatoria.

[b] Values are the similarities estimated by the equation proposed by Nei and Li (1979) for each *hrp*-related fragment restricted with either endonuclease *Cfo*I, *Hae*III, *Sau*3AI, and *Taq*I

highly related and they are monophyletic in the phylogenetic analysis with bootstrap resampling value of 80% (Fig. 5-2). Further, the similarity index between members of the clades 3 and 4 ranged from 0.96 to 1.00 for the *hrpB* region (Table 5-2).

Discussion

The phylogenetic analysis of the *hrp* genes of the xanthomonads has revealed a diverse evolutionary relationship for this region of the bacterial genome of these plant pathogens. The hypothesis of coevolution of the *hrp* region with the rest of the genome from a common bacterial ancestor is supported by comparison of either closely or distantly related plant pathogenic xanthomonads. For instance, the similarity of the two *hrp* regions examined in this study for the groups A and B of *X. campestris* pv. vesicatoria was less than 0.51. This genetic divergence determined for these two groups of *X. campestris* pv. vesicatoria is very similar to the values obtained when the entire genome was compared on the basis of DNA homology (Stall et al., 1994). Although these pathogens cause similar diseases on solanaceous plants, their *hrp* genes are genetically diverse at about the same extend as the rest of the genome. Moreover, these comparisons do not support the contention that there was a higher selective pressure for the *hrp* sequences than for other regions of the genome nor that a horizontal movement of the *hrp* region of the genome may have occurred between strains of the two groups of *X. campestris* pv. vesicatoria. Despite the genetic distance, the *hrp* genes of these two groups of *X. campestris* pv. vesicatoria are functionally complementary (Chapter 3). Similarly, the citrus pathogens *X. campestris* pv. citri and *X. campestris* pv. citrumelo also cause diseases on the same hosts though they are genetically distinct (Egel et al., 1991, Gabriel et al., 1989, Vauterin et al., 1991a). The analysis of the *hrp* genes revealed the same level of relatedness found when the entire genome were compared. Whereas the estimates of the similarity for the two regions of the *hrp* genes ranged from 0.41 to 0.61, the DNA-DNA hybridization relatedness ranged from 0.55 to 0.63 (Egel et al. 1991). In contrast, strains of *X. campestris* pv. citri canker A are genetically highly related to strains of *X. campestris* pv. malvacearum based on DNA-DNA hybridization studies (Egel et al., 1991). The analysis of the *hrp* genes also revealed a high similarity between these two groups of plant pathogenic xanthomonads with values ranging from 0.80 to 0.88 for both regions of the *hrp*-related sequences examined (Appendix D). Further, these two groups of *X. campestris* are monophyletic in regard to the evolution of the *hrp* genes (Fig. 5-1 and 5-2).

Additional evidence to support the coevolution of the *hrp* genes and the rest of the bacterial genome comes from the pathovars *X. campestris* pv. carotae, *X. campestris* pv. gardneri, and *X. campestris* pv. pelargonii. Although these xanthomonads have very distinct host ranges, they are genetically closely related based on DNA-DNA hybridization (Hildebrand et al. 1990, Palleroni et al., 1993). The phylogenetic analysis of the *hrp*-related sequences also supports the contention that these pathovars of *X. campestris* are monophyletic and genetically closely related in regard to the *hrp* genes. The examples discussed above not only point to a coevolution of the *hrp* genes and the rest of the genome from a common bacterial ancestor for certain plant pathogenic xanthomonads, but also substantiate the divergence between *hrp* genes and host speciation.

Another major finding of this study is the indication of lateral movement of the *hrp* genes between plant pathogenic xanthomonads. This hypothesis is supported by two lines of evidences, the presence of similar *hrp* gene cluster sequences in strains with divergent genetic background and the variability in the relatedness between strains depending on the *hrp* region examined. Strains of *X. campestris* pv. vesicatoria A belong to the same clade as the strains of *X. campestris* pv. citrumelo for both *hrp* genes examined (Fig. 5-1 and 5-2). Furthermore, the estimated genetic relatedness between strains of these two pathovars for the *hrp* genes regions ranged from about 0.71 to 0.96 (Appendix D) although these strains cause diseases on different hosts. On the contrary, DNA-DNA hybridization studies indicated that these two pathovars are not genetically closely related based on comparisons of the entire bacterial genome which was compared (Egel 1991). In fact, the relatedness between *X. campestris* pv. citrumelo and *X. campestris* pv. vesicatoria was about 0.58 which is close to the similarity determined between strains of *X. campestris* pv. citrumelo and *X. campestris* pv. citri discussed before (Egel, 1991, Egel et al., 1991). *X. campestris* pv. citrumelo has been reported occurring only in Florida (Schoulties et al., 1987). Although the origin of these strains is not very clear yet (Schoulties et al., 1987), the strains of this pathovar may have evolved from an endemic flora of xanthomonads that is present in Florida (Hartung and Civerolo, 1989). The analysis of the *hrp* genes of the strains of *X. campestris* pv. citrumelo revealed some degree of variability, but there is strong support for a monophyletic relationship among them. Furthermore, they are also closely related and monophyletic in regard to both regions of the *hrp* genes to strains of other pathovars of *X. campestris*, i.e. *X. campestris* pv. alfalfae, *X. campestris* pv. fici, *X. campestris* pv. poinsettiicola, *X. campestris* pv. pruni and *X. campestris* pv. vesicatoria A, and the strain X198 of *X. campestris*, which are also endemic to the same geographic area. A more extensive study of the genetic relationships among these pathogens also including nonpathogenic xanthomonads endemic to Florida may provide some important information on the origin of the strains of *X. campestris* pv. citrumelo.

Stronger evidence that supports the contention of lateral movement of the *hrp* genes among the plant pathogenic xanthomonads comes from the variability in the relatedness between strains for the two distinct *hrp* regions examined in this study. For instance, the *hrpC/D* region of *X. campestris* pv. vignicola is phylogenetically monophyletic and closely related to the homologous *hrp* region of *X. campestris* pv. bilvae, *X. campestris* pv. citri, *X. campestris* pv. glycines A, *X. campestris* pv. malvacearum, *X. campestris* pv. phaseoli B, *X. campestris* pv. phaseoli "fuscans", and *X. campestris* pv. vitians B (Fig. 5-2). The *hrpB* region of *X. campestris* pv. vignicola is also monophyletic, but it is closely related to the homologous *hrp* region of *X. campestris* pv. begoniae, *X. campestris* pv. dieffenbachiae, *X. campestris* pv. maculifoliigardeniae, *X. campestris* pv. manihotis, and *X. campestris* pv. phaseoli A (Fig. 5-1 and 5-2). In a similar way, the *hrpC/D*-related region of *X. campestris* pv. raphani was only 0.50 similar to the homologous region in *X. campestris* pv. campestris, *X. campestris* pv. armoraciae, and *X. campestris* pv. incanae (Table 5-2), whereas the *hrpB*-related region was more than 0.90 similar for the strains of these two groups (Table 5-2). Moreover, functional heterologous complementation of the *hrp* genes has been demonstrated for different xanthomonads (Chapter 3, Arlat et al., 1991, Bonas et al., 1991). Since there is no genetic and functional support regarding selection pressure in relation to the *hrp* genes among the plant pathogenic xanthomonads, the most likely hypothesis to explain the source of variability for these two regions of the bacterial genome of *X. campestris* pv. vignicola remains in the origin of the two *hrp* regions from distinct ancestors. The variability in the genetic relatedness for these two regions of the *hrp* genes of *X. campestris* pv. vignicola substantiate the contention that the *hrp* genes in some of the plant pathogenic xanthomonads may have evolved from distinct ancestors through lateral movement of genetic material.

An important conclusion from the phylogenetic studies is the divergence between *hrp* genes and host specificity. The *hrp* genes are essential for the development of disease on compatible hosts and hypersensitive reaction in both resistant host and nonhost plants (Willis et al., 1991). Previous work has demonstrated the functional conservation of the *hrp* genes and the lack of host speciation (Chapter 3, Arlat et al. 1991, Bonas et al., 1991). Although the *hrp* genes are necessary in the plant-pathogen interaction, other factors in the bacterial pathogen are likely to be involved in host speciation (Fenselau et al., 1992, Gough et al., 1992). Our results support the coevolution of the *hrp* genes with the rest of the bacterial genome through a common bacterial ancestor. Also, there are bases to support the hypothesis of horizontal movement of the *hrp* genes. Close relationship between plant pathogenic xanthomonads with different genetic background and variability in the relatedness of different regions of the *hrp* genes indicate distinct origins for different regions of the *hrp* genes, instead of evolution from a single ancestor. Since the lateral movement of genetic material between bacteria is a common and important mechanism in bacterial evolution (Krawiec and Riley, 1990), the coexistence in the same biological niche may have provided opportunities for the lateral transfer of the *hrp* region of the bacterial genome between xanthomonads. The *hrp* are functionally conserved among the xanthomonads (Chapter 3, Bonas et al., 1991, Fenselau et al., 1992), this also supports the absence of functional selectivity. The resemblance at the protein level of the *hrp* genes of the xanthomonads with genes involved in the secretion of pathogenicity factors in genetically distant organisms such as the animal pathogens of the genus

Yersinia (Fenselau et al 1992, Gough et al 1992) is also intriguing. If this means a convergent functional evolution or a common bacterial ancestry remains to be clarified. Furthermore the phylogeny of the *hrp* genes of plant pathogenic xanthomonads may provide a framework and a rational basis through which origins and differentiation of the xanthomonads may be assessed

CHAPTER 6

GENETIC ANALYSIS OF *hrp* RELATED DNA SEQUENCES OF *Xanthomonas campestris* STRAINS CAUSING DISEASES OF CITRUS

Citrus canker, caused by strains of *Xanthomonas campestris* pv. citri group A, represents an important problem for production of citrus worldwide (Civerolo, 1984). This disease is characterized by raised lesions on leaves, stems, and fruits. Strains of *X. campestris* pv. citri group A have a relatively wide host range and cause symptoms of various degrees in all commercial citrus varieties (Stall and Seymour, 1983). In severe cases, abscission of fruits and leaves may result (Civerolo, 1984, Stall and Seymour, 1983). Other xanthomonads that cause similar symptoms on citrus are strains of groups B and C of *X. campestris* pv. citri. They are of less importance than strains of *X. campestris* pv. citri group A and have comparatively limited host ranges. Citrus bacterial spot is another bacterial disease of citrus caused by a xanthomonad, and symptoms are similar to citrus canker with a few important differences (Schoulties et al., 1987). The pathogen, referred to as *X. campestris* pv. citrumelo (Gabriel et al., 1989), causes flat, watersoaked lesions to young leaves. Strains of *X. campestris* pv. citrumelo cause symptoms primarily on trifoliate orange (*Poncirus trifoliata*) and its hybrids, such as Swingle citrumelo (*Citrus paradisi* X *P. trifoliata*) (Graham and Gottwald, 1988).

Although *X. campestris* pv. citri and *X. campestris* pv. citrumelo cause similar diseases of citrus, there is evidence for differences between these pathovars. In addition to the pathogenicity differences listed above, *X. campestris* pv. citrumelo strains appear to be quite heterogeneous both genetically (Egel, 1991; Egel et al., 1991) and in aggressiveness (Graham and Gottwald, 1990) compared with *X. campestris* pv. citri. This has resulted in questions about the relationship of the bacterial spot pathogen to other pathovars of *X. campestris*. Several xanthomonads isolated from ornamental plants cause lesions similar to bacterial spot when artificially inoculated onto young citrus plants (Graham and Gottwald, 1991; Graham et al., 1990); they are also genetically similar to some strains of *X. campestris* pv. citrumelo (Egel et al., 1991; Graham et al., 1990). It was suggested that strains of *X. campestris* pv. citrumelo may represent other pathovars of *X. campestris* incidentally isolated from citrus (Graham et al., 1990) or strains of a xanthomonad that has a wide host range (Gabriel et al., 1988); alternatively it was suggested that the most weakly aggressive strains may be opportunistic strains which cause symptoms only when associated with injury (Egel, 1991).

These alternatives have not been resolved by studies of the genetics (Egel et al., 1991; Gabriel et al., 1988, 1989; Graham et al., 1990; Hartung and Civerolo, 1987, 1989; Vauterin et al., 1991b) or pathogenicity (Graham and Gottwald, 1990; Graham et al., 1990) of these strains. The genetics of pathogenicity, however, might favor one of the above hypotheses. An excellent candidate for examination is the hypersensitivity reaction and pathogenicity (*hrp*) gene cluster responsible for pathogenicity reaction on susceptible hosts and a hypersensitive reaction on resistant hosts or on nonhosts plants (Willis et al., 1991). The *hrp* gene cluster has been characterized in several bacterial plant pathogens, such as *Pseudomonas syringae* pv. phaseolicola (Lindgren et al., 1986), *P. solanacearum* (Boucher et al., 1987), *Erwinia amylovora* (Beer et al., 1991), and *X. campestris* pv. vesicatoria (Bonas et al., 1991). The *hrp* gene cluster of *X. campestris* pv. vesicatoria consists of at least 25 kb of genomic DNA and polymorphism of restriction fragments of a homologous DNA sequence occurs among pathovars of *X. campestris* (Bonas et al., 1991). Opportunistic xanthomonads, which produce limited symptoms in susceptible hosts and no hypersensitive reaction in nonhosts, do not possess DNA similar to an *hrp* gene cluster (Stall and Minsavage, 1990).

The genomic similarity of strains of *X. campestris* pv. citrumelo has been investigated (Egel et al., 1991; Gabriel et al., 1988, 1989; Graham et al., 1990; Hartung and Civerolo, 1987, 1989; Vauterin et al., 1991b), but examination of the similarity between *hrp* clusters of these strains adds information on the comparative genetics of pathogenicity. Similar *hrp* gene clusters among strains with relatively divergent genetic backgrounds might indicate a similar origin of pathogenicity, and dissimilar *hrp* gene clusters would support the hypothesis that many strains of *X. campestris* involved in the citrus bacterial spot disease are diverse. The similarity of the *hrp* gene in strains of *X. campestris* pv. citri and *X. campestris* pv. citrumelo was investigated by amplifying and restricting two DNA fragments of the *hrp* complementation groups B and C/D (Chapter 3; Bonas et al., 1991), which are highly conserved among several pathovars of *X. campestris* (U. Bonas, personal communication).

Materials and Methods

Culture conditions

The strains of *X. campestris* used in this study and their sources are listed in Appendix A. All strains had previously been identified as members of *X. campestris* by fatty acid analysis (N. C. Hodge, personal communication). Citrus bacterial spot strains were rated for pathogenicity by Graham and Gottwald (1990) and by Graham et al (1990) All strains were streaked onto nutrient agar (Becton Dickinson, Cockeysville, MD), and single colonies were selected Nutrient broth cultures were grown 24 hours on a rotatory shaker (150 rpm) at 28°C Strains of *X. campestris* pv *citri* group B were grown no a sucrose based medium (Canteros de Echenique et al., 1985) Strains were stored on lima bean agar (Difco Detroit, MI) for short term storage and in sterile tap water at room temperature for long term storage

DNA isolation

The procedure described by Ausubel et al (1987) with minor modifications, was used to extract total genomic DNA Briefly bacterial cells were pelleted by centrifuging in an Eppendorf microcentrifuge (Brinkmann Instruments Inc , Westbury, NY) for 2 min at 16 000 *g* The pellet was washed in 1 ml of distilled water, pelleted again and resuspended in 567 µl of TE buffer (10 mM Tris-Cl, pH 8 0 1 mM EDTA, pH 8 0) Proteinase K (Boehringer Mannheim, Indianapolis, IN) and sodium dodecyl sulfate (SDS) (Sigma, St Louis, MO) were added for a final concentration of 100 µg/ml and 0 5%, respectively After incubation for 1 hour at 37°C sodium chloride and hexadecyltrimethyl ammonium bromide (Sigma) were added to each preparation for a final concentration of 0 7 M and 1%, respectively The preparations were incubated for 10 min at 65°C DNA was extracted with chloroform-isoamyl alcohol (24 1) The samples were hand shaken continuously and gently for 10 min and centrifuged for 5 min at 16,000 *g* A second extraction was accomplished by adding phenol-chloroform-isoamyl alcohol (25 24 1) and centrifuging as described above DNA was precipitated by adding 0 6 volumes of isopropanol and incubating for 30 min at -20°C The samples were centrifuged for 20 min at 16,000 *g* The DNA pellet obtained was washed with 1 ml of 70% ethanol and centrifuged again The DNA was dried under vacuum for 20 min and then the pellet was redissolved in 100 µl of TE and stored at 4°C

DNA amplification

The two sets of oligonucleotide primers used in this study were designed based on nucleotide sequences of the *hrp* gene cluster of *X. campestris* pv. vesicatoria (U. Bonas, personal communicatino). The two primers RST2 and RST3 delineate an 840-bp region and primers RST21 and RST22 delineate a 1,075 bp region of the *hrp* complementation groups B and C/D of *X. campestris* pv. vesicatoria, respectively (Chapter 3). Oligonucleotide primers were synthesized with a model 394 DNA Synthesizer (Applied Biosystems, Foster City, CA) by the DNA Synthesis Laboratory, University of Florida, Gainesville.

DNA was amplified in a total volume of 50 µl. The reaction mixture contained 5 µl of 10X buffer (500 mM KCl, 100 mM Tris Cl [pH 9.0 at 25°C], 1% Triton X-100), 1.5 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (Boehringer Mannheim), 25 pmol of each primer, and 2.5 units of *Taq* polymerase (Promega, Madison, WI). The amount of template DNA added was 100 ng of purified total bacterial DNA. The reaction mixture was covered with 50 µl of light mineral oil. A total of 30 amplificatino cycles were performed in an automated thermocycler (MJ Research, Watertown, MA). Each cycle consisted of 30 s of denaturation at 95°C, 30 s of annealing at 62°C, and 45 s of extension at 72°C for the primers RST2 and RST3 and 30 s at 95°C, 45 s at 61°C and 1.5 min at 72°C, respectively, for the primers RST21 and RST22. The last extension step was extended to 5 min.

Amplified DNAs were detected by electrophoresis in 0.9% agarose gels in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.2) at 5 V/cm of gel (Sambrook et al., 1989.) After being stained with 0.5 µg of ethidium bromide per ml, the gel was photographed over a UV transilluminator (Fotodyne Inc., New Berlin, WI) with type 55 Polaroid film (Polaroid, Cambridge, MA).

Hybridization analysis

The identity of the amplified DNA fragments was further confirmed by hybridization analysis with an internal DNA probe for each fragment. Samples were electrophoresed in 0.7% agarose gel according to standard procedure (Sambrook et al., 1989.) The gel was then denatured in 0.4 N NaOH and 0.6 M NaCl for 30 min and neutralized for 30 min in 0.5 M Tris Cl and 1.5 M NaCl. The denatured DNA was transferred by the procedure of Southern (1975) to nylon membrane (Gene Screen Plus, Du Pont, Boston, MA). Hybridization was carried out at 68°C with 0.5X SSC and 0.1% w/v SDS. The internal probes consisted of a 271 bp insert of the plasmid pXV840 for the 840 bp fragment and a 335 bp insert of the plasmid pXV1075 for the 1,075-bp fragment (Chapter 3, Appendix A). Probes were labeled by the random primed (Feinberg and Vogelstein, 1983) incorporation of digoxigenin-labeled deoxyuridine-triphosphate (DIG-UTP) and detected by the use of the Genius Nonradioactive DNA Labeling and Detection kit (Boehringer Mannheim) as specified by the manufacturer.

Restriction endonuclease analysis of amplified DNA

Amplified DNAs were restricted with either endonuclease *CfoI*, *Hae*III, *Sau*3AI, or *TaqI* under conditions specified by the manufacturer (Promega). The restriction fragments were separated by electrophoresis in 4% agarose gels (3% NuSieve GTG and 1% Seakem GTG [FMC BioProducts, Rockland, ME]) in TAE buffer at 8 V/cm. Phage λ *Pst* I restricted DNA fragments were used as molecular weight standards. After being stained with 0.5 µg of ethidium bromide per ml for 40 min, the gels were destained in 1 mM $MgSO_4$ for 1 hr and then photographed over a UV transilluminator with type 55 Polaroid film.

Data analysis

DNA restriction fragment patterns were determined by direct comparison of the electrophoretic patterns of the DNA restricted with each of the four endonucleases. The codes 1 or 0 were assigned according to the presence or absence of each fragment, respectively. The resulting matrix was used to estimate the genetic relationships between strains based on the proportion of shared DNA fragments. The expected proportion of shared fragments (F) was calculated by the equation proposed by Nei and Li (1979): $F = 2n_{xy}/(n_x + n_y)$, where n_{xy} is the number of fragments shared between two strains and n_x and n_y are the total number of fragments for each strain. A computer program (Appendix B) written for the SAS system (SAS Institute Inc., Cary, NC) was used to estimate the proportion of shared fragments (F). The genetic divergence between strains was calculated as the estimate of the number of nucleotide substitutions per site ($\hat{\delta}$), based on the proportion of shared DNA fragments (Nei and Li, 1979). The iterative method proposed by Nei (1987) was used to estimate the number of nucleotide substitutions per site using a computer program written for the SAS system (Appendix C).

Relationships among strains were studied based on phylogenetic analysis using the BOOT and KITSCH programs from the PHYLIP computer package (Felsenstein, 1991). For the BOOT program, the restriction fragment data encoded 0 or 1 were used as input for reconstruction of an unrooted phylogenetic tree by using the Wagner parsimony criterion. No assumptions were made regarding the ancestral character state, and the pathovar *X. campestris* pv. maculifoliigardeniae strain X22j was taken as the outgroup to infer the topology of the phylogenetic tree. A total of 100 bootstrap samples were analyzed to determine the confidence intervals of the estimates of the inferred phylogenetic tree (Felsenstein, 1985, 1991). The KITSCH program was used to infer a rooted phylogenetic tree by the Fitch-Margoliash method (Felsenstein, 1991; Fitch, and Margoliash, 1967). The input data consisted of a distance matrix of pairwise estimates of the number of nucleotide substitutions per site, (δ), between strains, and negative branch was not allowed.

Results

DNA amplification

The 840- and 1,075-bp fragments of the *hrp* gene cluster in *X. campestris* pv. vesicatoria were successfully amplified with primers RST2 plus RST3 (Fig. 6-1) and RST21 plus RST22 (Fig. 6-2), respectively. The same size fragments were also successfully amplified from DNA of all strains of the other pathovars of *X. campestris* (Fig. 6-1 and 6-2). The DNA fragments were also amplified from 16 strains of *X. campestris* pv. citrumelo representing the three aggressiveness groups and from 19 strains of *X. campestris* pv. citri groups A, B, and C without variation in size (data not shown). The sequence similarity of the two DNA fragments amplified from strains of different pathovars of *X. campestris* to the *hrp* gene cluster of *X. campestris* pv. vesicatoria was further confirmed by Southern hybridization analysis. The amplified DNA fragments of the different strains of *X. campestris* hybridized to the respective internal probes specific for both *hrp* gene cluster fragments (data not shown).

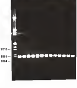


Fig 6-1 Amplification of the 840-bp fragment of the complementation group B of the *hrp* gene cluster of *Xanthomonas campestris* pv vesicatoria from strains of *X campestris* Lanes M, phage λ restricted with *Eco* RI and *Hind* III 1 to 3 strains F1, F6 and F100 of *X c* pv citrumelo respectively, 4, *X c* pv maculifoliigardeniae strain X22j 5 *X campestris* from *Strelitzia reginae* X198, 6, *X. c.* pv fici strain X151, 7 *X c* pv alfalfae strain 82-1, 8, *X c* pv bilvae strain XCB, 9 *X. campestris* from *Feronia* sp. strain XCF, 10 to 12, strains 9771, B84 and 339 of *X. c.* pv citri, respectively; 13, *X c.* pv vesicatoria strain 75-3 Molecular sizes are given in bases

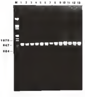


Fig 6-2 Amplification of the 1,075-bp fragment of the complementation groups C/D of the *hrp* gene cluster of *Xanthomonas campestris* pv vesicatoria from strains of *X campestris* Lanes M, phage λ restricted with *Eco* RI and *Hind* III, 1 to 3 strains F1, F6, and F100 of *X c* pv citrumelo respectively, 4, *X c* pv maculifoliigardeniae strain X22j 5 *X campestris* from *Strelitzia reginae* X198 6, *X c* pv fici strain X151 7, *X c* pv alfalfae strain 82-1 8 *X c* pv bilvae strain XCB 9, *X campestris* from *Feronia* sp strain XCF 10 to 12, strains 9771, B84 and 339 of *X c* pv citri, respectively, 13 *X c* pv vesicatoria strain 75 3 Molecular sizes are given in bases.

Restriction analysis

The 840- and 1,075 bp *hrp* gene cluster fragments amplified from strains of different pathovars of *X. campestris* were each restricted with either *Cfo*I, *Hae*III, *Sau*3AI, or *Taq*I. The banding patterns for each set of fragments from the pathovars of *X. campestris* included in this study were variable. The banding patterns of the 1,075 bp *hrp* gene cluster fragment amplified from strains of different pathovars of *X. campestris* restricted with the endonucleases *Cfo*I and *Hae*III are presented in Fig. 6-3. The banding patterns of the strains of *X. campestris* pv. citrumelo were very similar to the patterns obtained for *X. campestris* pv. vesicatoria strain 75-3 (Fig. 6-3). Also, *X. campestris* pv. alfalfa, *X. campestris* pv. fici and the strain X198 of *X. campestris* produced patterns similar to the strains of *X. campestris* pv. citrumelo with certain combinations of fragment-restriction endonucleases (Fig. 6-3). *X. campestris* pv. bilvae, *X. campestris* XCF$_1$ and the strains of *X. campestris* pv. citri also made up a group with very similar banding pattern (Fig. 6-3). On the other hand, *X. campestris* pv. maculifoliigardeniae had a more distinct restriction fragment profile (Fig. 6-3). Although variability was also observed in the banding patterns obtained with the endonucleases *Sau*3AI and *Taq*I, a characteristic pattern for each group or pathovar of *X. campestris* was less evident for these two endonucleases (data not shown). Similarly, restriction analysis of the 840-bp *hrp* fragment also produced a pattern of variation for the different strains of *X. campestris* (data not shown).

Sixteen strains of *X. campestris* pv. citrumelo, representing all three aggressiveness groups, were analyzed by restriction analysis of the amplified *hrp* fragments. The banding patterns of five strains of the highly aggressive group of *X. campestris* pv. citrumelo were identical to each other when restricted with either *Cfo*I (Fig. 6-4A), *Hae*III (Fig. 6-4B), *Sau*3AI or *Taq*I (data not shown). For certain

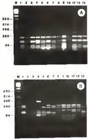

M 1 2 3 4 5 6 7 8 9 10 11 12 13
A
805
514
468
264
94
M 1 2 3 4 5 6 7 8 9 10 11 12 13
B
805
514
339
200
94

Fig 6-3 Restriction analysis of the 1,075 bp DNA fragment of the complementation groups C/D of the *hrp* gene cluster amplified from strains of *Xanthomonas campestris* and restricted with the endonucleases (A) *Hae*III and (B) *Cfo*I Lanes M, phage λ restricted with *Pst*I 1 to 3 strains F1, F6 and F100 of *X c* pv citrumelo respectively, 4, *X c* pv maculifoliigardeniae strain X22j, 5, *X campestris* from *Strelitzia reginae* strain X198, 6 *X c* pv fici strain X151, 7 *X c* pv alfalfae strain 82-1, 8 *X c* pv bilvae strain XCB, 9, *X campestris* from *Feronia* sp. strain XCF 10 to 12, strains 9771 B84 and 339 of *X c* pv citri respectively 13 *X c* pv vesicatoria strain 75-3 Molecular sizes are given in bases.

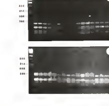


Fig. 6-4 Restriction analysis of the 1,075-bp DNA fragment of the complementation groups C/D of the *hrp* gene cluster amplified from strains of *Xanthomonas campestris* pv. citrumelo and restricted with the endonucleases (A) *Hae*III and (B) *Cfo*I. Lanes M, phage λ restricted with the *Pst* I; 1 to 5, highly aggressive strains F1, F54, F274, F361 and 3166, respectively; 6 to 11, moderately aggressive strains F6, F228, F311, F254, F348 and F378; 12 to 16, weakly aggressive strains F59, F86, F100, F306 and F94; 17, *X. c.* pv. vesicatoria strain 75-3. Molecular sizes are given in bases.

combinations of fragment and endonuclease, the restriction pattern of the highly aggressive strains were similar to some strains of the moderately or weakly aggressive groups, these include the 1 075-bp fragment from the weakly aggressive strain F100 restricted with *Cfo*I (Fig. 6-4A) and the fragment from the moderately aggressive strain F378 restricted with *Hae*III (Fig. 6-4B). However, the overall banding patterns of the combinations of two fragments and four endonucleases for the highly aggressive strains were different from the patterns obtained for the strains of the moderately and weakly aggressiveness groups of *X. campestris* pv. citrumelo. In contrast to the highly aggressive group, restriction fragment polymorphism was evident for the strains within the moderately and weakly aggressive groups of *X. campestris* pv. citrumelo (Fig. 6-4). The weakly aggressive strains F94, F100, and F306 had banding patterns almost identical to *X. campestris* pv. vesicatoria strain 75-3 (Fig. 6-4). Also, strains of the highly aggressive group of *X. campestris* pv. citrumelo had banding patterns similar to *X. campestris* pv. fici strain X151 (Fig. 6-3 and 6-4).

In contrast to the diversity of the moderately and weakly aggressive strains of *X. campestris* pv. citrumelo, strains of *X. campestris* pv. citri of the groups A, B, and C each produced characteristic restriction patterns (Fig. 6-5). The banding patterns of all strains of group A were identical when restricted with either *Hae*III (Fig. 6-5), *Cfo*I, *Sau*3AI, or *Taq*I (data not shown). Similarly, strains of groups B and C were also homogeneous within each group (Fig. 6-5), as well as between the two groups for the four endonucleases and the two *hrp* gene cluster fragments.

Genetic relationships of the *hrp* from different strains of *X. campestris*

Differences in the number of common restriction fragments from the amplified DNA of the *hrp* gene cluster indicated that there is variation in the relatedness of the

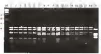


Fig. 6-5 Restriction analysis of the 1 075 bp DNA fragment of the complementation groups C/D of the *hrp* gene cluster amplified from strains of *Xanthomonas campestris* pv citri restricted with the endonuclease *Hae*III Lanes M: phage λ restricted with the *Pst*I, 1-6, group A strains 9771 3340 9760-2, 3213, T1, and 115 A, 7 to 13 group B strains B64 B69 B80 B84 B93 B94 and B148, 14 to 18 group C strains 338, 339, 340 341 and 342, 19 *X. c* pv vesicatoria strain 75 3 Molecular sizes are given in bases

hrp genes of the different strains of *X. campestris*. The genetic divergence between strains was estimated based on the data for 106 restriction fragments obtained from the combination of two *hrp* gene cluster fragments and four endonucleases. A pairwise matrix of the genetic distances, δ, was calculated for the 18 distinct banding patterns (Table 6-1). *X. campestris* pv. citri 339 was included in the genetic analysis as a representative of group C, although the restriction banding patterns of the *hrp* fragments were identical to those of the strains of group B of *X. campestris* pv. citri. The largest genetic divergence value was 0.082 nucleotide substitution per site between *X. campestris* pv. maculifoliigardeniae X22j and *X. campestris* pv. citri 9771 of group A (Table 6-1). However, most of the estimates of nucleotide substitutions per site are smaller than 0.05, which is considered the upper limit to give accurate estimates of genetic distance based on restriction fragment data (Nei 1987).

Strains of *X. campestris* pv. citrumelo that represent all three aggressiveness groups exhibited nine different restriction patterns of the *hrp* fragments that were divergent from 0.002 to 0.022 nucleotide substitution per site (Table 6-1). Similarly, strains of *X. campestris* pv. citri groups A, B, and C showed a low genetic divergence for the *hrp* genes, ranging from 0.000 to 0.014 nucleotide substitution per site (Table 6-1). As mentioned above, the banding patterns of the strains of *X. campestris* pv. citri groups B and C were identical to each other for all combinations of *hrp* gene cluster fragments and restriction endonucleases. On the other hand, the *hrp* genes of strains of *X. campestris* pv. citrumelo were very poorly related to the ones of *X. campestris* pv. citri, with divergence ranging from 0.050 to 0.064 nucleotide substitution per site (Table 6-1).

Table 6-1 Genetic divergence matrix for 19 strains of *Xanthomonas campestris* based on the estimates of the number of nucleotide substitutions per site in two fragments related to the *hrp* gene cluster *X. campestris* pv. vesicatoria.

	X. campestris pv. *citrumelo*[a]									*X. campestris* pv. *citri*[b]			Other strains of *X. campestris*						
	High	Moderate				Weak				A	B	C							
	F1	F6	F311	F348	534	F86	F94	F100	F306	9771	884	339	82-1	XCB	XCF	X22j	X198	X151	75-3
F1	–	0.020	0.009	0.015	0.010	0.014	0.016	0.022	0.020	0.057	0.055	0.055	0.011	0.045	0.041	0.060	0.019	0.004	0.014
F6			0.009	0.015	0.019	0.012	0.012	0.018	0.010	0.057	0.063	0.063	0.007	0.056	0.057	0.052	0.015	0.018	0.018
F311			–	0.015	0.012	0.008	0.010	0.014	0.012	0.057	0.055	0.055	0.007	0.045	0.044	0.049	0.011	0.007	0.012
F348				–	0.010	0.010	0.014	0.016	0.016	0.056	0.061	0.061	0.011	0.045	0.046	0.058	0.017	0.009	0.018
534						0.011	0.017	0.017	0.015	0.051	0.050	0.050	0.014	0.036	0.037	0.045	0.018	0.010	0.015
F86							0.007	0.011	0.009	0.050	0.051	0.051	0.006	0.037	0.038	0.048	0.008	0.012	0.012
F94							–	0.005	0.002	0.057	0.055	0.055	0.012	0.046	0.044	0.052	0.012	0.014	0.005
F100								–	0.007	0.064	0.058	0.058	0.016	0.049	0.047	0.052	0.016	0.010	0.011
F306									–	0.057	0.055	0.055	0.014	0.046	0.044	0.052	0.014	0.016	0.023
9771										–	0.014	0.014	0.059	0.008	0.013	0.082	0.052	0.051	0.057
884											–	0.000	0.061	0.012	0.011	0.080	0.051	0.049	0.055
339												–	0.061	0.012	0.011	0.080	0.051	0.049	0.055
82-1													–	0.048	0.049	0.065	0.011	0.011	0.018
XCB														–	0.002	0.074	0.039	0.038	0.046
XCF															–	0.075	0.040	0.038	0.044
X22j																–	0.051	0.060	0.055
X198																	–	0.017	0.018
X151																		–	0.012
75-3																			–

[a] High, Moderate, and Weak denote aggressiveness groups.

[b] A, B and C denote native groups.

The relatedness of the *hrp* genes of strains of the citrus pathogens *X. campestris* pv. citrumelo and *X. campestris* pv. citri to the *hrp* gene cluster of some other pathovars of *X. campestris* was also investigated. The *hrp* gene cluster of strains of *X. campestris* pv. alfalfae 82-1, *X. campestris* pv. fici X151, and *X. campestris* pv. vesicatoria 75-3 and *X. campestris* X198 were closely related to *hrp* genes of *X. campestris* pv. citrumelo, and the genetic divergence ranged from 0.004 to 0.023 nucleotide substitution per site (Table 6-1). However, strains of *X. campestris* pv. citri were much less related to these four strains of *X. campestris*, with genetic divergence ranging from 0.049 to 0.061 nucleotide substitutions per site (Table 6-1). The *hrp* genes of strains of *X. campestris* pv. citri were highly related to *X. campestris* pv. bilvae XCB and *X. campestris* XCF. The genetic divergence of the *hrp* genes of *X. campestris* pv. citri from the genes of these strains of *X. campestris* ranged from 0.011 to 0.018 nucleotide substitution per site (Table 6-1). Moreover, *X. campestris* pv. maculifoliigardeniae strain X22j has *hrp* genes not highly related to any of the xanthomonads from citrus, with a genetic divergence ranging from 0.046 from *X. campestris* pv. citrumelo strain 534 to as high as 0.082 from *X. campestris* pv. citri group A (Table 6-1).

The restriction fragment data of the *hrp* genes encoded 0 or 1 and the distance matrix (Table 6-1) were used to construct phylogenetic trees based on a parsimony criterion by using the BOOT program and a distance method by using the KITSCH program of the PHYLIP computer package (Felsenstein, 1991), respectively. Although the general topology is slightly different, the phylogenetic trees inferred by using two different approaches of tree reconstruction showed very similar branching patterns for the major clades (Fig. 6-6 and 6-7). The branching pattern obtained with the BOOT program is unrooted, although the strain *X. campestris* pv. maculifoliigardeniae X22j

Fig. 6-6 Unrooted tree for 19 strains of *Xanthomonas campestris* inferred from restriction analysis data of DNA fragments related to the complementation groups B and C/D of the *hrp* genes of *X. campestris* pv. vesicatoria generated by the BOOT procedure from the PHYLIP computer package by using the Wagner parsimony criterion. Numbers at each node indicate the bootstrap percentages from 100 samples. Bootstrap values less than 50 indicate that the assemblage is not well supported by the data.

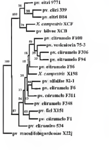
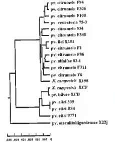

pv. citri 9771
56
98
pv. citri 339
pv. citri B84
100
79
X. campestris XCF
pv. bilvae XCB
59
pv. citrumelo F100
pv. vesicatoria 75-3
16
35
pv. citrumelo F306
33
pv. citrumelo F94
15
pv. citrumelo F86
100
X. campestris X198
25
44
pv. alfalfae 82-1
pv. citrumelo F6
17
pv. citrumelo F311
54
13
pv. citrumelo F348
69
pv. fici X151
87
pv. citrumelo F1
pv. citrumelo 534
pv. maculifoliigardeniae X22j

Fig. 6-7 Rooted tree for 19 strains of *X. campestris* inferred from restriction analysis data of DNA fragments related to the complementation groups B and C/D of the *hrp* genes of *X. campestris* pv vesicatoria generated by the KITSCH procedure from the PHYLIP computer package by using the Fitch Margoliash method


pv. citrumelo F94
pv. citrumelo F306
pv. citrumelo F100
pv. vesicatoria 75-3
pv. citrumelo 534
pv. citrumelo F348
pv. fici X151
pv. citrumelo F1
pv. citrumelo F86
pv. alfalfae 82-1
pv. citrumelo F311
pv. citrumelo F6
X. campestris X198
X. campestris XCF
pv. bilvae XCB
pv. citri 339
pv. citri B84
pv. citri 9771
pv. maculifoliigardeniae X22j
.030 .025 .020 .015 .010 .005 0
Genetic distance

was taken as the outgroup to infer the topology of the tree (Fig. 6-6). The strains of *X. campestris* can be divided into three major clades based on the *hrp* genes, with *X. campestris* pv. maculifoliigardeniae X22j as the sole member of one clade (Fig. 6-6). The second clade is the largest one and comprises all strains of *X. campestris* pv. citrumelo, *X. campestris* pv. alfalfae, *X. campestris* pv. fici, *X. campestris* pv. vesicatoria, and *X. campestris* from *S. reginae* (Fig. 6-6). The third clade is formed by all the strains of *X. campestris* pv. citri, *X. campestris* pv. bilvae, and a strain of *X. campestris* from *Feronia* sp. (Fig. 6-6). The assemblage of these phylogenetic clades is highly supported by the bootstrap values (Fig. 6-6). Furthermore, *X. campestris* pv. maculifoliigardeniae X22j was indeed chosen as an appropriate outgroup to evaluate the relationships among *X. campestris* strains causing disease on citrus on the basis of the *hrp* genes. The rooted tree obtained by the KITSCH program, selected among 1908 trees examined, also indicates that *X. campestris* pv. maculifoliigardeniae X22j is basal to the remainder *X. campestris* strains included in this study (Fig. 6-7).

The inferred phylogenetic trees seem to support the hypothesis that the *hrp* gene cluster of *X. campestris* pv. citrumelo strains from the three aggressiveness groups are monophyletic and closely related to other pathovars of *X. campestris*, including *X. campestris* pv. alfalfae, *X. campestris* pv. fici, and *X. campestris* pv. vesicatoria. Similarly, the monophyly of the *hrp* genes of *X. campestris* pv. citri, *X. campestris* pv. bilvae, and *X. campestris* XCF is also supported (Fig. 6-6 and 6-7).

Discussion

Strains of all pathovars of *X. campestris* included in this study have an *hrp* gene cluster on the basis of hybridization of genomic DNA with the *hrp* gene cluster from *X.*

campestris pv. vesicatoria (Egel, 1991) and on the basis of amplification of *hrp* fragments with oligonucleotide primers specific for the complementation groups B and C/D of the *hrp* genes of *X. campestris* pv. vesicatoria (Chapter 3). This is particularly significant in regard to the strains associated with citrus bacterial spot disease. Despite differences in the pathogenic characteristics of those strains (Graham and Gottwald, 1990; Graham et al., 1990), the presence of an *hrp* gene cluster supports the pathogenic nature of those bacterial strains. If they were opportunistic xanthomonads (Gitaitis et al., 1987), they would lack an *hrp* region (Bonas et al., 1991; Stall and Minsavage, 1990).

Information about the similarity of the *hrp* genes of the bacteria causing diseases of citrus was obtained from restriction enzyme patterns of amplified fragments of the *hrp* gene cluster. Although the DNA fragments amplified with the two sets of primers were of the same size for all the strains of *X. campestris*, characteristic restriction banding patterns for each bacterial group occurred with the 840- and 1,075-bp fragments. Complementation groups B and C/D of the *hrp* gene cluster, from which the fragments were amplified, are considered to be highly conserved among plant pathogenic xanthomonads (U. Bonas, personal communication). Therefore, the homology of the restriction enzyme fragments from amplified *hrp* genes should furnish valid relationships among these pathogens. These relationships were determined by a phylogenetic analysis.

The phylogenetic analysis based on the *hrp* gene cluster showed polyphyletic relationships of the strains of *X. campestris* causing disease in citrus. This suggests that the *hrp* gene cluster may have evolved independently in these strains of *X. campestris*. This evolution could be convergent or parallel. The analysis is based on the assumption that the differences in restriction sites in the *hrp* gene cluster region were due to nucleotide substitutions and not to insertion or deletion of DNA sequences. In fact, this assumption is supported by the fact that no apparent length variation was observed in the two DNA fragments amplified from all strains of *X campestris* In support of the phylogenetic analysis presented here is the monophyletic nature of the *hrp* gene cluster of bacterial pathogens with different genetic backgrounds causing disease in different hosts The phylogenetic grouping presented here also correlates with genetic analyses based on DNA-DNA hybridization, fingerprinting, and conventional restriction fragment length polymorphism (Egel 1991 Egel et al , 1991 Gabriel et al., 1988, 1989, Graham et al 1990 Hartung and Civerolo 1987 1989, Kubicek et al , 1989) The data presented supports the concept of a group of causal microorganisms of citrus bacterial spot disease which are closely related yet represent a variety of different genotypes

Two different groups of *X campestris* pv citri were distinguished by restriction enzyme analysis of the amplified fragments of the *hrp* gene cluster The strains of group A produced identical patterns for all fragment-endonuclease combinations. Similarly all strains within the groups B and C also produced identical restriction banding patterns. The banding patterns for group A strains were different from the patterns of group B and C strains, however This substantiates other reports of the relative genetic uniformity of the strains of the A, B and C groups of *X campestris* pv citri based on restriction analysis of the entire genome by using genomic fingerprinting pulse-field electrophoresis or restriction fragment length polymorphisms with random DNA probes (Egel, 1991, Egel et al 1991 Gabriel et al. 1988 1989, Graham et al , 1990 Hartung and Civerolo 1987) The two groups of *X campestris* pv citri have about 60% DNA homology (Egel et al 1991) The major difference from previous work is that in our studies specific homologous regions of the bacterial genome the *hrp* gene cluster were compared. Nevertheless, the results of the genetic analysis of the *hrp* related regions are consistent with those obtained when the entire genome was randomly examined. From this and the previously cited research, the two groups of strains within *X. campestris* pv. citri should probably be differentiated at some taxonomic level.

Two groups of *X. campestris* pv. citrumelo were also distinguished after restriction enzyme digestion of the amplified *hrp* fragments. Strains of the highly aggressive group were very uniform for all fragment-endonuclease combinations and had a characteristic restriction banding pattern. On the other hand, the moderately and weakly aggressive groups of the bacterium had diverse restriction banding patterns for both amplified *hrp* fragments. This concurs with previous studies of the moderately and weakly aggressive groups of *X. campestris* pv. citrumelo (Egel, 1991; Egel et al., 1991; Gabriel et al., 1988, 1989; Hartung and Civerolo, 1987, 1989; Kubicek et al., 1989). Even with genetic diversity among the moderately and weakly aggressive strains, all strains included under *X. campestris* pv. citrumelo are 80% similar using DNA-DNA hybridization (Egel, 1991; Egel et al., 1991). As with strains of *X. campestris* pv. citri, the two groups of strains of *X. campestris* pv. citrumelo should also be distinguished at the taxonomic level.

The results presented in this work demonstrate that strains of *X. campestris* causing disease in citrus can be reliably differentiated and identified by restriction analysis of amplified fragments related to the *hrp* gene cluster. The number of strains examined were small, but the genetic diversity within different pathovars of *X. campestris* could be assessed. The reliable identification of the citrus pathogen using DNA amplification will greatly facilitate disease diagnosis, as well as ecological and epidemiological studies. Furthermore the use of oligonucleotide primers for the *hrp* gene cluster region provide certainty for identification of strains *X. campestris* that may not be possible with other methods

CHAPTER 7

EVALUATION OF A DNA AMPLIFICATION APPROACH FOR DETECTION AND IDENTIFICATION OF PLANT PATHOGENIC XANTHOMONADS ASSOCIATED WITH PEPPER AND TOMATO SEEDS

Bacterial spot caused by *X. campestris* pv. vesicatoria is one of the most important diseases for pepper and tomato productions worldwide (Jones et al., 1991; Stall, 1993). The disease is characterized by necrotic lesions on leaves, stems, and fruits. In warm and rainy weather, the disease is usually severe and may cause heavy defoliation of the plants that results to reduced yield (Pohronezny et al., 1986). Furthermore, diseased fruits are usually commercially depreciated and may not be suitable for fresh market (Cox, 1966; Pohronezny et al., 1983). Bacterial spot is also of major concern in the certification program in the transplant industry in southern USA where producers are expected to provide disease-free transplants (Gitaitis et al., 1987, 1992).

Control of bacterial spot has been hampered by many factors. No bactericide is completely effective to control the disease and tolerance to bactericides, such as streptomycin and copper compounds, is widespread among strains of *X. campestris* pv. vesicatoria (Marco and Stall, 1983; Thayer and Stall, 1961). This has lead to reduced efficacy of bactericide sprays for the control of the disease in the field. Sources of resistance to bacterial spot were identified in pepper (Sowell and Dempsey, 1977) and tomato (Scott and Jones, 1989) and have been used in breeding programs to develop horticulturally desirable pepper and tomato cultivars. However, sudden shifts in races of *X. campestris* pv. vesicatoria can overcome the resistanceand poses a major concern for the use of such resistance commercially (Pohronezny et al , 1992) The causal bacterium may spread from diseased to healthy plants by wind- blown water, clipping of plants, and aerosols (Gitaitis et al , 1992, Jones et al , 1991, Volcani, 1969) Furthermore the bacterium may overwinter on tomato volunteers and diseased plant debris (Jones et al., 1986) Nevertheless, exclusion of the pathogen from pepper and tomato growing areas is still one of the main control measures Consequently, the use of pathogen free seeds and transplants has become an important part of the strategy for control of bacterial spot (Gitaitis et al , 1992 Jones et al., 1991)

X campestris pv vesicatoria has been reported to be in association with tomato and pepper seeds (Bashan et al 1982, Gardner and Kendrick 1921, 1923, Jones et al., 1986, Sharon et al , 1982) and symptoms of bacterial spot were observed in seedlings from contaminated seed lots (Gardner and Kendrick, 1923 Higgins, 1922). However, the detection and identification of *X campestris* pv vesicatoria associated with seeds is still a problem with the methods available The semiselective media developed specifically for *X campestris* pv vesicatoria (Gitaitis et al , 1991, McGuire et al., 1986, Sijam et al 1991) may still support the growth of strains of other pathovars of *X campestris* as well as nonpathogenic xanthomonads and saprophytes associated with seeds These bacteria may have cultural characteristics similar to *X campestris* pv vesicatoria (Gitaitis et al 1987, 1991 McGuire and Jones, 1989, McGuire et al , 1986, Sijam et al. 1991) Therefore, additional biological biochemical, or physiological tests are required to determine with certainty the identity of the xanthomonads recovered (Gitaitis et al 1991 McGuire and Jones, 1989) Serological tests with monoclonal and polyclonal antibodies have also been examined for specific identification of plant pathogenic xanthomonads, including *X campestris* pv vesicatoria (Benedict et al., 1990 O'Brien et al., 1967). However, the heterogeneous nature of antigenic determinants in immunogens of the xanthomonads poses a major limitation for the use of polyclonal antibodies. For instance, polyclonal antibodies developed for strains of *X. campestris* pv. vesicatoria cross-reacted with different plant pathogenic xanthomonads (O'Brien et al., 1967). In contrast, strains of *X. campestris* pv. vesicatoria are serologically diverse and no single polyclonal or monoclonal antibody has been obtained that reacts to all strains of this pathogen (Benedict et al., 1990; Jones et al., 1993b; Stall et al., 1994). More recently, nucleic acid based techniques have been examined for specific detection of *X. campestris* pv. vesicatoria (Garde and Bender, 1991; Jones et al., 1993b). Garde and Bender (1991) developed DNA probes for detection of copper resistant *X. campestris* pv. vesicatoria, but the copper region from *X. campestris* pv. vesicatoria may hybridize to other plant pathogens, such as the tomato pathogen *Pseudomonas syringae* pv. tomato (Voloudakis et al., 1993).

The objective of this study was to examine a procedure based on the amplification of DNA fragments related to the *hrp* gene cluster of *X. campestris* pv. vesicatoria (Chapter 3) for the specific detection and identification of plant pathogenic xanthomonads associated with pepper and tomato seeds. The sensitivity and specificity of the polymerase chain reaction (PCR) technique were determined by the detection of *X. campestris* pv. vesicatoria in artificially and naturally contaminated seeds lots. The reliability of the technique for detection of *X. campestris* pv. vesicatoria was further investigated by comparison with the dilution plating on semiselective media and detection by ELISA. Furthermore, plant pathogenic xanthomonads isolated from pepper and tomato seeds were tentatively identified based on the analysis of the restriction pattern produced from amplified DNA fragments related to the *hrp* genes.

Materials and Methods

Bacterial strains and culture conditions

The bacterial strains isolated from pepper and tomato materials which were used in this study and their sources are listed in Table 7.1. All strains had been identified previously as members of the *Xanthomonas* by fatty acid analysis (N. C. Hodge, personal communication). All strains were streaked on nutrient agar (Becton Dickinson) and single colonies were selected. Nutrient broth cultures were grown 24 hr on a rotatory shaker (150 rpm) at 28 °C. Strains were stored in sterile tap-water at room temperature.

Seed sources and artificial infestation

Pepper and tomato seeds were obtained from fruits or were provided by commercial seed companies. Naturally infested seeds of pepper and tomato were obtained by extracting seeds from diseased fruits or were provided by the Georgia Department of Agriculture at Tifton, GA. In some experiments, bacterial suspensions of *X. campestris* pv. vesicatoria 75-3 was added to the seed wash mixture at the appropriate concentrations before ultrasonication.

Plant material and plant inoculations

All plants were maintained in a growth chamber at 28-30°C during inoculation and incubation. The pepper cultivar Early Calwonder and the tomato cultivar, Bonny Best were used in the pathogenicity tests. In pathogenicity tests, fully expanded leaves of plants were inoculated with bacterial suspensions by infiltrating the bacteria into the intercellular spaces by using a 1 ml plastic syringe with a 27 gauge needle. The concentration of the inoculum was approximately 5 X 10^8 CFU/ml in sterile tap water determined by measuring the optical density in a Spectronic 20 spectrophotometer (Bausch and Lomb Inc, Rochester NY) Plant reactions were scored over a period of several days.

Recovery of bacteria from seeds and seedlings

The bacterial fraction was recovered from seeds and seedlings by using the ultrasonication technique (Haefele and Webb 1982 Morris, 1985) Two and one half grams of seeds, or ten grams of aerial parts of seedling washings were washed in 20 ml or 150 ml of phosphate buffer (8 5 mM K_2HPO_4 7 5 mM KH_2PO_4, 0 02% Tween 20, pH 7 0), respectively, for 20 min in a model B 22-4 ultrasonic cleaner (Branson Cleaning Equipment Co , Shelton CT) Bacterial populations were determined by the dilution plate count method (Taylor, 1962) Dilutions were made from the washings in sterile tap-water and 0 1 ml aliquots were plated onto yeast-extract nutrient agar (YNA) (Schaad and Stall, 1988) or Tween medium B (McGuire et al , 1986), or both *X campestris* pv vesicatoria was determined by the presence of circular, raised, and yellow colonies surrounded by a lipolytic halo on Tween medium B (McGuire et al , 1986 McGuire and Jones, 1989) Aliquots of the seed and seedling washings were also processed and used in the DNA amplification and ELISA assays.

Extraction of DNA from bacteria and plant washings

Total genomic DNA of each bacterial strain was isolated by phenol-chloroform extraction and ethanol precipitation essentially as described by Ausubel et al (1987) Bulk DNA extracts were obtained from seed and seedling washings by using a modification of the method for DNA extraction described by Ausubel et al (1987)

Aliquots of the seed or seedling washings were transferred to 1.5 ml microfuge tubes, and sodium ascorbate (Sigma, St. Louis, MO) and insoluble polyvinypolypyrrolidone (PVPP) (Sigma) were added for a final concentration of 0.2 M and 0.1%, respectively. These concentrations of PVPP and sodium ascorbate were used throughout this study, unless otherwise stated. Prior to use, the PVPP was acid washed by the procedure described by Holben et al. (1988). The samples were homogenized by vortexing. The homogenate was pelleted by centrifuging in an Eppendorf microcentrifuge (Brinkmann Instruments Inc., Westbury, NY) for 2 min at 16,000 *g*. The pellet was resuspended in 567 µl of TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0). Proteinase K (Boehringer Mannheim, Indianapolis, IN) and sodium dodecyl sulfate (SDS) (Sigma) were added for a final concentration of 100 µg/ml and 0.5%, respectively. After incubation for 1 hour at 37°C, sodium chloride and hexadecyltrimethyl ammonium bromide (Sigma) were added to each preparation for a final concentration of 0.7 M and 1%, respectively. The preparations were incubated for 10 min at 65°C. DNA was purified by treatment with chloroform-isoamyl alcohol (24:1). The samples were vortexed and centrifuged for 5 min at 16,000 *g*. A second purification was accomplished by adding phenol-chloroform-isoamyl alcohol (25:24:1) and centrifuging as described above. DNA was precipitated by adding 0.6 volumes of isopropanol and incubating for 30 min at -20°C. The samples were centrifuged for 20 min at 16,000 *g*. The DNA pellet obtained was washed with 70% ethanol and centrifuged again. After drying, the pellet was redissolved in 50 µl of TE buffer and stored at 4°C.

DNA amplification

Three sets of oligonucleotide primers selected from the nucleotide sequence of the *hrp* gene cluster of *Xanthomonas campestris* pv. vesicatoria (Ulla Bonas, personal communication) were used in this study. Primers RST2 plus RST3 delineated an 840-bp fragment, RST9 plus RST10 delineated a 355-bp fragment, and RST21 plus RST22 delineated a 1,075-bp fragment. The primers map to the complementation groups *hrpB* and *hrpC/D* of the *hrp* gene cluster of *X. campestris* pv. vesicatoria (Chapter 3). Oligonucleotide primers were synthesized with a model 394 DNA Synthesizer (Applied Biosystems, Foster City, CA) by the DNA Synthesis Laboratory, University of Florida, Gainesville.

DNA fragments were amplified in a reaction mixture of 50 µl containing 5 µl of 10X buffer (500 mM KCl, 100 mM Tris Cl [pH 9.0 at 25°C], 1% Triton X 100), 1.5 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (Boehringer Mannheim), 25 pmol of each primer, 1.25 units of *Taq* polymerase (Promega, Madison, WI). The amount of template DNA added was 100 ng of purified bacterial DNA whereas for the seed and seedling samples a volume of 10 µl of the DNA extract was added, unless otherwise stated. The reaction mixture was overlaid with 50 µl of light mineral oil. Thirty amplification cycles were performed in an automated thermocycler PT-100-60 (MJ Research, Watertown, MA) according to the following profiles: 30 s of denaturation at 95°C, 30 s of annealing at 62°C, and 45 s of extension at 72°C for the primers RST2 plus RST3, 30 s of denaturation at 95°C, 30 s of annealing at 52°C, and 45 s of extension at 72°C for the primers RST9 plus RST10, 30 s of denaturation at 95° C, 40 s of annealing at 61°C, and 45 s of extension at 72°C for the primers RST21 plus RST22. For all three profiles, the initial denaturation step was 5 min at 95°C, and the last extension step was extended to 5 min. Amplified DNA fragments were detected by electrophoresis in 0.9% agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.2) according to standard procedures (Sambrook et al., 1989).

Restriction endonuclease analysis

The *hrp*-related fragments amplified from different bacterial strains were restricted with the frequent-cutting endonucleases *Cfo*I, *Hae*III, *Sau*3AI, or *Taq*I, according to conditions specified by the manufacturer (Promega). The restricted fragments were separated by electrophoresis in 4% agarose gels (3% NuSieve and 1% Seakem GTG [FMC BioProducts]) in TAE buffer at 8 V/cm. Phage λ *Pst*I-restricted DNA fragments were used as molecular standards. The gel was stained with 0.5 µg of ethidium bromide per ml for 40 min and then destained in 1 mM $MgSO_4$ for 1 hr and photographed over a UV transilluminator with type 55 Polaroid film. The restriction pattern obtained was compared to the standard banding profiles established for the different groups of plant pathogenic xanthomonads (Chapter 4).

ELISA assay

An ELISA procedure that was developed for detection of low populations of *X. campestris* pv. vesicatoria was used (Somodi et al. 1993). Aliquots of the seed washings were transferred to microfuge tubes and an equal amount of EDTA/lysozyme lysis buffer (2.0 g KH_2PO_4, 11.5 g Na_2PO_4, 0.14 g EDTA disodium, 0.02 g thimerosal, 0.2 g lysozyme (Sigma), 1 l deionized water) was added. The samples were homogenized by vortexing and incubated for at least 16 hr at room temperature.

Immulon 2 (Dynatech Laboratories, Chantily, VA) flat bottom 96-well microtiter plates were coated with a polyclonal antibody developed against *X. campestris* pv. vesicatoria 75-3 and incubated at 4°C overnight. The coating buffer and phosphate buffered saline (PBS) were used as described by Clark and Adams (1977). All further incubations were for 2 hr at 37°C. The microtiter plates were washed with a solution containing 0.8% NaCl and 0.1% Tween 20 in deionized water (NTrinse) (G

C Somodi personal communication) Plates were then blocked with 1% bovine albumin (Sigma, A 9647) in PBS and shaken to remove excess liquid Aliquots of 100 µl of each sample in EDTA/lysozyme lysis buffer were added to three wells on the microtiter plate and incubated The microtiter plates were rinsed with NTrinse and a monoclonal antibody (2H10) prepared against *X campestris* pv vesicatoria 75-3 was added to all wells and the plates were incubated The microtiter plates were rinsed again with NT rinse, and alkaline phosphatase conjugated goat antimouse A 1047 (Sigma) was applied to the plate The microtiter plates were incubated and then rinsed with the final washing buffer Tris-buffered saline (1 51 g of Tris-base 2 19 g NaCl, final volume 250 ml with deionized water pH 7 5) four times. Substrate and amplifier were added according to the instructions specified by the manufacturer of the ELISA Amplification System (Gibco BRL Gaithersburg MD) Readings were made at A_{492} 15 min after addition of the amplifier with an EAR400 AT plate reader (SLT Labinstruments Austria).

Fatty acid analysis

The fatty acid profiles of the strains of *X campestris* isolated from pepper and tomato seeds and seedlings were determined A single colony of each strain was transferred from a nutrient agar culture to trypticase soy broth agar (TSBA) Approximately 40 mg of cells of a 24 h growth at 28°C was collected for analysis. The bacterial fatty acids were derivatized to their methyl esters (Miller 1982) and separated by gas chromatography (Sasser 1990) and identified with the Microbial Identification System software (version 3 80 MIDI Newark, DE) The similarity index and best match for the strains based on fatty acid analysis data were determined by using the MIDI software

Table 7-1 Phenotypic and genetic characterization of strains of *Xanthomonas campestris* associated with pepper and tomato seed and seedling

Strain	Origin	Reaction on plants		Amplification of *hrp* fragment		Tentative identification		Source[a]
		pepper	tomato	*hrpB*	*hrpC/D*	*hrp* analysis[b]	FAA[c]	
SP1 92	pepper seed	[d]	HR	+[e]	+	pv campestris[f]	pv campestris (0.820)	GOK
SP101 92	pepper seed	-	HR	+	+	pv campestris	pv campestris (0.463)	GOK
SP268 92	pepper seed	-		-	-	nd	pv armoraciae (0.703)	GOK
SP290 92	pepper seed	-	HR	-	-	na	pv pruni (0.852)	GOK
3118-GP	pepper seed	+	HR	+	+	pv vesicatoria A	pv vesicatoria (0.735)	GOK
7502-2FS1	pepper seed	-	HR	-	-	na	pv raphani (0.677)	JD
157	pepper seed	HR	HR	+	+	nd	pv juglandis (0.644)	WW
T1083	tomato seed					na	pv celebensis (0.051)	WW
524A-1	pepper seed	HR	HR	+	+	pv campestris	pv armoraciae (0.768)	WW
524A/2	pepper seed	HR	HR	+	+	pv campestris	pv armoraciae (0.812)	WW
75-0-3	tomato seed	HR	+	+	+	pv vesicatoria C	pv manihotis (0.835)	WW
7B 0-1	tomato seed	HR	+	+	+	pv vesicatoria C	pv fici (0.773)	WW
T1087	tomato seed	HR	HR	+	+	pv citrumelo	pv poinsettiicola (0.789)	WW
1 A-0-1	pepper seed	+	+	+	+	pv vesicatoria A	pv vesicatoria (0.745)	WW
P996	pepper seed	-	-	-	-	na	pv raphani (0.581)	WW
DM-1	pepper seed	-	+	+	+	nd	pv juglandis (0.618)	WW
LM-1	tomato seed	-	HR		(+)	nd	pv malvacearum (0.644)	WW
639-6/FS1	pepper seed	-	-	-	-	na	nd	JW
639-6/FS2	pepper seed	-	-	-	-	na	nd	JW

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Table 7.1—*Continued*

Strain	Origin	Reaction on plants		Amplification of *hrp* fragment		Tentative identification		Source
		pepper	tomato	*hrpB*	*hrpC/D*	*hrp* analysis	FAA	
9310	tomato seedling	HR	+	+	+	pv vesicatoria B	pv manihotis (0.723)	DAM
9311	tomato seedling	HR	+	+	-	nd	pv campestris (0.778)	DAM
140A-d1	tomato seed	HR	HR	+	+	pv raphani	pv campestris (0.897)	DAM
140A-d2	tomato seed	HR	HR	+	+	pv raphani	pv campestris (0.911)	DAM
T-93-23	tomato transplant	HR	+	+	+	pv vesicatoria B	pv alfalfae (0.707)	DAM
724-4	tomato seed	HR	HR	+	-	nd	pv vesicatoria (0.816)	DAM
P93-22	pepper seedling	-	-	-	-	na	pv pruni (0.799)	GOK
T93-09	tomato seedling	HR	HR	+	+	pv raphani	pv campestris (0.715)	GOK
T93-12A	tomato seedling			-	-	na	pv zinniae (0.856)	GOK
VSE069	tomato seed	HR	+	+	+	pv vesicatoria B	pv vesicatoria (0.698)	MD
VSE070	tomato seed	HR	+	+	+	pv vesicatoria B	pv vesicatoria (0.620)	MD
VSE071	tomato seed	HR	+	+	+	pv vesicatoria B	pv vesicatoria (0.718)	MD
BSA1	pepper seed	HR	HR	+	+	nd	pv corylina (0.628)	JW
BSA2	pepper seed	HR	HR	+	+	nd	pv corylina (0.619)	JW
BSA3	pepper seed	HR	HR	(+)	(+)	nd	pv pruni (0.671)	JW
BSA4	pepper seed	HR	HR	+	+	pv citrumelo	pv fici (0.765)	JW
BSA5	pepper seed	HR		-	-	na	pv cannae (0.598)	JW
BSA6	pepper seed	HR		-	-	na	pv cannae (0.586)	JW
BSA7	pepper seed	HR	HR	-	-	na	pv pruni (0.832)	JW
BSA8	pepper seed	HR	HR	(+)	+	pv carotae	pv carotae (0.730)	JW

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Table 2-1—*Continued*

Strain	Origin	Reaction on plants		Amplification of *hrp* fragment		Tentative identification		Source
		pepper	tomato	*hrpB*	*hrpC/D*	*hrp* analysis	FAA	
BSA9	pepper seed	HR	HR	(+)	+	pv. carotae	pv. carotae (0.824)	JW
BSA10	pepper seed	HR	HR	(+)	+	pv. carotae	pv. carotae (0.903)	JW
BSA11	pepper seed	HR	HR	(+)	+	pv. carotae	pv. carotae (0.852)	JW
BSA12	pepper seed	HR	HR	(+)	+	pv. carotae	pv. carotae (0.767)	JW
BSA13	pepper seed		HR	(+)	+	pv. carotae	pv. carotae (0.753)	JW
BSA14	pepper seed	HR	HR	(+)	+	pv. carotae	pv. carotae (0.577)	JW
BSA15	pepper seed	HR	HR	(+)	+	pv. carotae	pv. carotae (0.748)	JW
BSA16	pepper seed	-	-	(+)	+	pv. carotae	pv. carotae (0.841)	JW
BSA17	pepper seed	-	-	(+)	+	pv. carotae	pv. carotae (0.868)	JW
BSA18	pepper seed	HR	HR	-		na	pv. malvacearum (0.736)	JW
BSA19	pepper seed	HR	HR	-		na	pv. malvacearum (0.720)	JW
BSA20	pepper seed	-	HR	(+)	+	pv. carotae	pv. carotae (0.789)	JW
BSA21	pepper seed	-	-	(+)	+	pv. carotae	pv. carotae (0.905)	JW
BSA22	pepper seed			(+)	+	pv. carotae	pv. carotae (0.809)	JW
BSA23	pepper seed	HR	HR	(+)	+	pv. carotae	pv. carotae (0.782)	JW
BSA24	pepper seed		HR	(+)	+	pv. carotae	pv. carotae (0.779)	JW
BSA25	pepper seed			(+)	+	pv. carotae	pv. carotae (0.752)	JW

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Table 7.1—*Continued*

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[a] DAM, D. A. Maddox, Seed Testing of America, Longmont, CO; GOK, G. O'Keefe, Department of Agriculture, State of Georgia, Tifton, GA; JD, J. Dodson, Woodland Research Station, Petoseed, Woodland, CA; JW, J. Watterson, Woodland Research Station, Petoseed, Woodland, CA; MD, M. Dene, Washington State University, Pullman, WA; WW, W. Wiebe, Rogers NK, Woodland, CA

[b] Identification of the strain was based on comparison of the *hrp* profile established to the different groups of plant pathogenic xanthomonads (see Chapter 4)

[c] Identification based on the MIDI TSBA Library (version 3.80; Microbial Identification System software, Newark, DE). Number in parenthesis is the similarity index

[d] -, no *hrp*-related fragment amplification; +, *hrp*-related fragment amplification; (+) weak *hrp*-related fragment amplification

[e] -, no reaction; +, pathogenic reaction; HR, hypersensitive reaction

[f] Strains of *X. campestris* pv. campestris could not be distinguished from strains of *X. campestris* pv. armoraciae by the restriction analysis of both *hrp* fragments (see Chapter 4)

[g] na, not applicable; nd, not determined

Results

Development of a DNA amplification approach for detection of plant pathogenic xanthomonads in seed extracts

Cells of *X. campestris* pv. vesicatoria were detected in extracts of tomato seeds by amplification of an 840-bp DNA fragment of the *hrp* gene cluster. The fragment was amplified from bulked DNA preparations obtained from the extracts to which cells of strain 75-3 were added (Fig. 7-1 and 7-2). The 355-bp *hrpB* fragment was also amplified from the same samples (data not shown). The *hrp* fragments were amplified from the samples processed with and without the presence of PVPP and sodium ascorbate (Fig. 7-1 and 7-2). Similar results were also obtained with other pepper and tomato seed lots (data not shown). The two reagents have been added to environmental and plant samples in order to assure the extraction of nucleic acids with the quality required for consistent enzymatic amplification of DNA fragments (Holben et al., 1988; Minsavage et al., 1994). The addition of PVPP, or sodium ascorbate, or both at different concentrations to the seed washings apparently did not interfere with the recovery of DNA, nor the amplification of the *hrp* fragments by polymerase chain reaction (Fig. 7-1 and 7-2). Other methods of sample preparation, such as the quick approach of DNA isolation (Kawasaki, 1990), did not produced consistent results in the amplification of *hrp* fragments for the detection of *X. campestris* pv. vesicatoria present in pepper and tomato seed washings (data not shown).

Investigation was carried out to further examine the quality of the DNA extracted from tomato seed washings with sodium ascorbate and PVPP added to the final concentration of 0.2 M and 0.1%, respectively, or without these reagents for amplification of the *hrp* fragments. Aliquots of 5 µl, 10 µl, and 15 µl of DNA extracted from tomato seed washings containing cells of *X. campestris* pv. vesicatoria

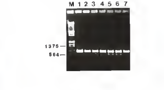

Fig 7-1 Amplification of the 840 bp fragment of the complementation group B of the *hrp* gene cluster from seeds of tomato containing 10^6 CFU/ml of *Xanthomonas campestris* pv vesicatoria 75 3 and different amounts of PVPP Lanes. M, phage λ restricted with *Eco*RI and *Hind*III, 1 no PVPP nor sodium ascorbate, 2 no PVPP 3, 1% of PVPP 4, 2% of PVPP, 5 3% of PVPP 6, 4% of PVPP, 7, 5% of PVPP Molecular sizes are given in bases

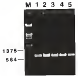

Fig. 7.2. Amplification of the 840-bp fragment of the complementation group B of the *hrp* gene cluster from seeds of tomato containing 10^5 CFU/ml of *Xanthomonas campestris* pv. vesicatoria 75-3 and different amounts of sodium ascorbate. Lanes: M, phage λ restricted with *EcoRI* and *HindIII*, 1, no sodium ascorbate nor PVPP, 2, no sodium ascorbate, 3, 0.02 M of sodium ascorbate, 4, 0.2 M of sodium ascorbate, 5, 2 M sodium ascorbate. Molecular sizes are given in bases.

75 3 were added to the PCR reaction mixture for amplification of the *hrpB* fragment with primers RST2 and RST3 The 840-bp *hrpB* fragment was amplified from all different samples and no significant inhibition of the amplification reaction was observed (Fig 7 3)

Sensitivity of the detection procedure

The sensitivity of the DNA amplification procedure for detection of cells of *X campestris* pv vesicatoria in preparations of tomato seed extracts was about 10^2 CFU/ml when the primers RST9 and RST10 were used for amplification of the 355 bp *hrpB* fragment (Fig 7-4 Table 7-2) The minimum detection level of *X. campestris* pv vesicatoria was usually 10 to 100 times higher when the primers RST2 and RST3 were used for amplification of the 840 bp *hrpB* fragment (Fig 7-4 Table 7-2) The lower sensitivity of the primers RST2 and RST3 for detection of *X. campestris* pv vesicatoria was also observed for other tomato and pepper seed lots (data not shown) Despite the differences in sensitivity both sets of primers produced specific amplification of the *hrp* fragments from seed extracts containing different concentration of *X campestris* pv vesicatoria (Fig 7-4) In comparison to ELISA, the DNA amplification procedure was at least 100 to 1000 times more sensitive for detection of *X campestris* pv vesicatoria when cells were added to the seed preparation (Table 7 2) The detection level of *X campestris* pv vesicatoria by ELISA was greater than 10^5 CFU/ml (Fig 7-4 Table 7-2).

Detection of plant pathogenic xanthomonads in naturally contaminated seed lots

Investigations were conducted to determine the feasibility of the DNA amplification procedure for detection of the presence of plant pathogenic

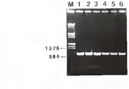

Fig 7-3 Amplification of the 840 bp fragment of the *hrp* gene cluster of *Xanthomonas campestris* pv vesicatoria 75 3 from seeds of tomato by using sodium ascorbate and PVPP (lanes 1, 2, and 3) and without these reagents (lanes 4, 5 and 6) Lanes: M phage λ restricted with *Eco*RI and *Hind*III 1 and 4 5 µl of DNA extract; 2 and 5 10 µl of DNA extract 3 and 6 15 µl of DNA extract. Molecular sizes are given in bases

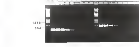

Fig 7-4 Amplification of the 355-bp (Lanes 2 to 10) and 840-bp (Lanes 12 to 20) fragments of the complementation group B of the *hrp* gene cluster from samples of tomato seeds containing different concentration of *Xanthomonas campestris* pv vesicatoria 75-3 added to the seed washings Lanes 1 and 11, phage λ restricted with *Eco*RI and *Hind*III 2 and 12, 2 6 X 10^8 CFU/ml 3 and 13, 2 9 X 10^7 CFU/ml 4 and 14, 0 9 X 10^6 CFU/ml, 5 and 15, 3 4 X 10^5 CFU/ml, 6 and 16 1 0 X 10^4 CFU/ml, 7 and 17, 3 3 X 10^3, CFU/ml 8 and 18 3 0 X 10^2 CFU/ml, 9 and 19, 4 0 X 10^1 CFU/ml 10 and 20 no bacteria added Concentration of *X campestris* pv vesicatoria in the seed washings was determined by plating on Tween B medium. Molecular sizes are given in bases.

Table 7-2 Sensitivity of ELISA and DNA amplification procedures for detection of *Xanthomonas campestris* pv. vesicatoria in tomato seeds

Sample	Total population of bacteria on YNA (CFU/ml)	*X. campestris* recovered on Tween B medium (CFU/ml)	ELISA A_{nm492}[a]	Amplification of *hrp* fragment[b]	
				355 bp	840 bp
1	2.9×10^{8}	2.0×10^{8}	2.79 ± 0.01	+[c]	+
2	7.3×10^{7}	6.7×10^{7}	2.09 ± 0.24	+	+
3	5.4×10^{6}	4.6×10^{6}	0.51 ± 0.01	+	+
4	5.6×10^{5}	5.5×10^{5}	0.21 ± 0.01	+	+
5	6.8×10^{4}	6.8×10^{4}	0.16 ± 0.02	+	+
6	5.8×10^{3}	5.2×10^{3}	0.16 ± 0.01	+	+
7	1.7×10^{2}	1.4×10^{2}	0.17 ± 0.01	+	-
8	2.6×10^{2}	7.0×10^{1}	0.17 ± 0.01	-	-
9	1.5×10^{2}	not detected	0.16 ± 0.01	-	-

[a] Values are the average of the spectrophotometer readings of three wells.

[b] Aliquots of 300 µl and 600 µl of the seed washings were used in the ELISA assay and for extraction of DNA, respectively. In the DNA amplification assay was used 10 µl of DNA template.

[c] - no *hrp* fragment amplification, + *hrp* fragment amplification.

xanthomonads in naturally contaminated seeds Plant pathogenic xanthomonads in pepper and tomato seed lots were detected consistently by amplification of the *hrpB* fragments in DNA extracted from washes of 11 seed lots out of the 15 tested (Table 7-3) The technique specificaly amplified the expected DNA fragments despite the background bacterial microflora of more than 10^5 CFU/g of seed (Fig 7-5, Table 7-3) The primers RST9 and RST10 failed to produce amplification of the 355-bp *hrp*-related fragment from the pepper seed lots SP135 92 and PK 1-PF though amplification of the 840-bp *hrp* related fragment indicated the presence of plant pathogenic xanthomonads in these seeds (Table 7-3) This is not surprising because the set of primers RST9 and RST10 seem to allow DNA amplificaton only from strains of a limited number of pathovars of *X campestris* (Chapter 3)

Attempts to recover plant pathogenic xanthomonads from different seed lots by plating on general and semiselective media was unsuccessful Whereas no colonies typical of xanthomonads on both media used, YNA and Tween B were found with the seed lots containing low bacterial population a large background bacterial microflora in extracts from other seed lots prevented the identification of any possible xanthomonads that may have grown However the presence of viable plant pathogenic xanthomonads in 5 seed lots was confirmed by detection of the bacteria in seedlings from seeds (Table 7 3) The detection of the plant pathogenic xanthomonads in the seedlings was accomplished by isolating the bacteria from lesions in the leaflets, or by amplification of the *hrp*-related fragments from bulked DNA extracted from washings of the aerial part of the seedlings grown under greenhouse conditions Furthermore, the presence of viable plant pathogenic xanthomonads in the pepper seed lots SP2 92, SP66 92 SP124 92 SP133 92 SP135 92 and SP306 92 had been determined previously (G O'Keefe, personal communication)

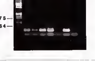

Fig 7-5 Amplification of the 355 bp DNA fragment related to the complementation group B of the *hrp* gene cluster of *Xanthomonas campestris* pv vesicatoria from DNA extracted from different pepper seed lots Lanes M, phage λ restricted with *Eco*RI and *Hin*dIII, 1 SP2 92, 2, SP66 92, 3, SP124 92, 4, SP133 92, 5, SP135 92, 6, SP306 92, 7, Greenhouse grown, 8, Jupiter Molecular sizes are given in bases.

Table 7-3 Detection of plant pathogenic xanthomonads in naturally infected pepper and tomato seeds.

Seed sample	Total population of bacteria on YNA (CFU/g seed)	Recovery of xanthomonads		Amplification of *hrp* fragment^a		Source of the seed^b
		Tween B medium	Grow out^c	355 bp	840 bp	
Pepper						
SP2 92	3.2×10^2	+^{d}	-	+	+	GOK
SP66 92	1.9×10^3	+	-	+	+	GOK
SP124 92	3.2×10^2	+	+	+	+	GOK
SP133 92	$>2.4 \times 10^5$		+	+	+	GOK
SP135 92	1.6×10^2	+	-	+	+	GOK
SP306 92	0.8×10^2	+	+	+	+	GOK
Jupiter	4.0×10^2		-		-	RST
Greenhouse grown	5.4×10^2	+	-	(+)	(+)	RST
PK-1-PF	$>4.0 \times 10^3$	+	-	+	+	JFW
PK 2 PF	$>4.0 \times 10^3$		+	+	+	JFW
PL 3-P	$>4.0 \times 10^3$		-	+	+	JFW
Tomato						
#51-1b	4.0×10^3	-	+	+	-	MM
Diseased fruits	1.1×10^7	-	+	+	nd	RST
Marglobe #100016	4.6×10^3	-	-		nd	RST
Manalucie	1.0×10^3	-	+	-	nd	RST

Continued on the following page

Table 7 3—*Continued*

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[a] Amplification of fragments related to the *hrpB* of *Xanthomonas campestris* pv vesicatoria by 30 cycles of polymerase chain reaction

[b] GOK, G O Keefe Department of Agriculture State of Georgia, Tifton, GA, JFW J F Wang, The Asian Vegetable Research and Development Center Tainan, Taiwan MM M Meadows Rogers NK Naples, FL, RST R E Stall University of Florida, Gainesville

[c] Presence of plant pathogenic xanthomonads was determined by isolation of bacteria or by detection by amplification of *hrp*-related fragment from diseased tissue

[d] - negative result, + positive result (+) weak positive result nd, not determined

Tentative identification of *X. campestris* associated with seeds and seedlings of pepper and tomato

The identity of 56 strains of *Xanthomonas* from seeds and seedlings of pepper and tomato was investigated by examining the reaction on plants and by analyzing DNA sequences related to the *hrp* genes and fatty acid composition (Table 7-1). The strains seem to comprise a very diverse group of xanthomonads that includes plant pathogenic and nonplant pathogenic bacteria. Some of the strains were pathogenic on plants of Bonny Best tomato and were identified as *X. campestris* pv. vesicatoria on the basis of the *hrp* fragment analysis, with the exception of the strains DM 1 and 9311 (Table 7-1). In the case of the latter strains *hrp*-related fragments were amplified, but the restriction analysis of the fragments were not done. The strains SP268.92, P996, 639-6/FS1, 639-6/FS2, T93-12A, BSA5, and BSA6 did not show any reaction on plants and they failed to produce the 840- and 1,075 bp *hrp*-related fragments in the DNA amplification assay (Table 7-1). Although these strains have some degree of similarity to different pathovars of *X. campestris* on the basis of fatty acid composition (Table 7-1), they resemble the opportunistic xanthomonads that have been reported previously in association with tomato and pepper transplants (Gitaitis et al., 1987, 1992). The remainder of strains form a very diverse group of potential plant pathogenic xanthomonads, as determined by the hypersensitive reaction on plants, or by the presence in their genome of a region similar to the *hrp* genes, or by both criteria (Table 7-1).

The identification of these strains of *Xanthomonas* based on the *hrp* analysis was performed by comparison of the restriction profile generated by digestion of the *hrp* fragment amplified from the strains with frequent cutting endonucleases (Fig. 7-6) to the pattern established for the different groups of plant pathogenic xanthomonads (Chapter 4). The restriction analyses allowed the identification of groups of strains of *X. campestris* pv. vesicatoria (Table 7-1). Two strains were identified to the group A, five to

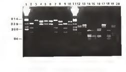

Fig. 7-6 Restriction analysis of the 1 075-bp DNA fragments related to the *hrpC/D* complementation group and amplified from strains of *Xanthomonas campestris* isolated from pepper and tomato seed and seedling, and restricted with the endonucleases *Taq*I (Lanes 2 to 10) and *Hae*III (Lanes 12 to 20) Lanes 1 and 11, phage λ restricted with *Pst*I; 2 and 12, 157-3 and 13, 524A-1, 4 and 14, 75-0-3, 5 and 15 7B-0-1, 6 and 16 T1087, 7 and 17, 524A-2, 8 and 18 1-A-0-1 9 and 19 DM-1 10 and 20, *X campestris* pv vesicatoria 75-3 Molecular sizes are given in number of bases

the group B of *X. campestris* pv. vesicatoria, and two to group C (Table 7-1). The fatty acid analysis did not identify the groups of strains and strains 75-0-3 and 9310 were similar to the fatty acid library of *X. campestris* pv. manihotis, and the strains T-93-23 and 7B-0-1 that were very similar to *X. campestris* pv. alfalfae and *X. campestris* pv. fici, respectively (Table 7-1). Most of the strains that produced hypersensitive reaction on pepper and tomato were identified as *X. campestris* pv. armoraciae, *X. campestris* pv. campestris, *X. campestris* pv. carotae, or *X. campestris* pv. raphani with a high degree of agreement in the identification by analyses of DNA sequences related to the *hrp* genes and by fatty acid composition (Table 7-1). Fifteen strains isolated from pepper seeds were identified as *X. campestris* pv. carotae by analyses of DNA sequences related to the *hrp* genes and fatty acid composition, though some variability was observed on the reaction on plants (Table 7-1). The restriction analysis of the *hrp*-related fragments amplified from the strains SP290 92, 157, T1083, DM-1, LM-1, 9311, 724-4, BSA1, BSA2, and BSA3 produced pattern different from those determined for 50 different groups of plant pathogenic xanthomonads (Chapter 4), or the yield obtained in the DNA amplification was too low for restriction fragment length polymorphism analysis. Therefore, the identification of these strains on the basis of the restriction profile of the *hrp*-related fragments to pathovar or group of *X. campestris* was not possible.

Discussion

There is an increased need for specific and sensitive methods for detection of plant pathogenic bacteria associated with seeds and other plant propagative materials (Miller and Martin, 1988; Schaad, 1982; Saettler et al., 1989). Based on the results presented here, the amplification and analysis of DNA sequences related to the *hrp* genes can be a useful tool for the specific detection and identification of plant pathogenic xanthomonads. *X. campestris* pv. vesicatoria was readily detected in preparations containing cells added to pepper and tomato seed extracts and in seed washings obtained from naturally contaminated pepper and tomato seeds. The sensitivity of the method of DNA amplification ranged from about 10^2 to 10^3 CFU/ml of seed washings. This level was 100 to 1000 times higher than the level obtained with ELISA, and it is certainly comparable to the levels obtained with the most sensitive techniques available for detection of bacteria (Pickup, 1991; Saettler et al., 1989). Furthermore, the specificity of the method allowed the detection of plant pathogenic xanthomonads against a background bacterial micro flora larger than 10^7 CFU/g of seed. This level of non-target bacterial population may have prevented the detection of the pathogen by plating in general and semiselective media.

X. campestris pv. vesicatoria was detected in DNA extracted from seed washings containing the reagents PVPP and sodium ascorbate and from preparations without these reagents. Although the *hrp* fragments were amplified from DNA extracted from seed samples without adding reagents to prevent potential inhibitors, the addition of PVPP and sodium ascorbate in the seed washings before nucleic acid extraction may be needed to assure the necessary purity of the DNA for amplification. The presence of inhibitors have been a major concern in the extraction of high quality DNA from environmental and plant samples (Holben et al., 1988; Mansavage et al., 1994; Steffan et al., 1988). Nevertheless, the addition of these two reagents to the seed washings before DNA extraction apparently did not interfere with the yield of DNA fragments obtained in the amplification of the *hrp* fragments.

A basic feature of the approach for recovering bacterial DNA used here is the initial separation of intact bacterial cells from the seed washings by centrifugation. This is followed by lysis of the cells and purification of the DNA recovered. This cell extraction method may be less efficient than the direct lysis method that involves the release and extraction of DNA without prior separation of the cells from the original matrix (Ogram et al., 1988, Steffan et al., 1988). However, the cell extraction method is likely to ensure exclusion of extracellular DNA that may be present in the sample and may produce DNA of higher quality (Holben et al., 1988, Steffan et al., 1988). In fact, inconsistent results in the detection of *X. campestris* pv. vesicatoria were obtained with the quick approach of DNA isolation which employs direct lysis of the bacterial cells in the original extraction matrix.

The procedure of amplification of DNA sequences of the *hrp* genes was highly sensitive and specific for detection of plant pathogenic xanthomonads in DNA preparations from seeds washes. Based on dilution plating counts, *X. campestris* pv. vesicatoria could be detected in preparations containing less than 10^2 CFU added to seed extracts (Table 7.2). However, this level of sensitivity may be improved further by including a step for concentration of the cells in the sample to enhance the recovery of bacterial cells (Roth, 1989, Schaad, 1982). The concentration step concomitantly concentrates unwanted saprophytic microflora. Although the presence of a large background microflora is a major limitation in the detection of plant pathogenic xanthomonads in pepper and tomato seeds by planting on general or even semiselective media (Gitaitis et al., 1991, McGuire et al., 1986), the specificity of the DNA amplification procedure is likely to overcome this kind of problem. In fact, *X. campestris* pv. vesicatoria was detected in naturally contaminated seed lots containing background bacterial microflora larger than 10^7 CFU/g of seeds, whereas plating on semiselective medium failed to recover the xanthomonads (Table 7.2). Nevertheless, the transmission tests confirmed the presence of viable cells of plant pathogenic xanthomonads in 5 seed lots out of the 11 which produced positive results in the DNA amplification procedure. This is not surprising because *X. campestris* pv. vesicatoria has the capability to survive for long periods of time in seeds and to initiate disease (Bashan et al., 1982; Gardner and Kendrick, 1921, 1923).

A diverse group of xanthomonads was found associated with pepper and tomato seeds. The majority of the 56 strains examined have the basic characteristics of plant pathogenic xanthomonads. They produced pathogenic or hypersensitive reaction on plants in pathogenicity tests, and DNA sequences related to the *hrp* genes were amplified from their genome. On the contrary, seven strains did not produce reaction on plants, and they failed to produce DNA sequences similar to the *hrp* genes in DNA amplification assay. These strains resemble the opportunistic xanthomonads that have been described in association with pepper and tomato transplants (Gitaitis et al., 1987, 1992). Among the plant pathogenic xanthomonads, only nine strains were positively identified as *X. campestris* pv. vesicatoria, the pepper and tomato pathogen (Table 7.1). Another group included potential plant pathogenic strains that were not pathogenic to pepper and tomato but instead produced a hypersensitive reaction on these plants. Based on the analyses of DNA sequences related to *hrp* genes and fatty acid composition, they were highly related to different pathovars of *X. campestris* but not to *X. campestris* pv. vesicatoria. Furthermore, the identification of the strains based on restriction profiles of the DNA sequences related to the *hrp* genes agreed very closely with the identification by fatty acid analysis. Some of these strains were identified as *X. campestris* pv. campestris though their pathogenicity to cabbage was not determined. Strains similar to *X. campestris* pv. campestris and pathogenic to cabbage have been reported previously on pepper and tomato transplants (Gitaitis et al., 1987). Other strains were similar to *X. campestris* pv. raphani which has also been found commonly in association with pepper and tomato seeds (Gitaitis et al. 1987) Strains of this pathovar have a large host range and may cause disease on pepper and tomato plants (Gitaitis et al 1987, White, 1930) Furthermore outbreaks of a bacterial spot on tomato transplants in southern USA have been associated to strains that have genetic and phenotypic characteristics similar to *X. campestris* pv raphani (R E Stall personal communication). Another common intriguing point is the similarity of fifteen strains isolated from pepper seeds to *X campestris* pv carotae Both restriction analysis of the DNA sequences related to the *hrp* genes and fatty acid composition agreed in the identification of these strains as *X campestris* pv carotae (Table 7-1) I am unaware of any previous report of the association of strains similar to *X campestris* pv carotae with pepper seeds

The presence of a diverse xanthomonad community on pepper and tomato seeds certainly poses a major concern for health inspection in seed certification programs Whereas some of the xanthomonads are probably nonpathogenic to plants, others are pathogenic to pepper and tomato The latter were identified as *X campestris* pv vesicatoria based on pathogenicity tests, analysis of DNA sequences related to the *hrp* genes, and fatty acid composition. However a third group of xanthomonads that includes potential plant pathogenic bacteria was also identified These strains, tentatively identified as belonging to different pathovars of *X campestris* cause hypersensitive reaction or no reactions on pepper and tomato plants under artificial inoculation However the role that these strains may play in a bacterial disease syndrome on pepper and tomato plants remains to be clarified Furthermore, the association of such a diverse group of bacterial pathogens with pepper and tomato seeds is a major problem with the procedures currently available for their detection and identification These strains grow on semiselective media developed for the pepper and tomato pathogen *X campestris* pv vesicatoria (Gitaitis et al 1987 1991 McGuire et al 1986) and they may cause certain reactions on plants under artificial inoculations. Therefore, additional studies are necessary to determine the role of these xanthomonads found in the seeds of pepper and tomato in the subsequent development of bacterial diseases on seedlings and plants.

In conclusion, a procedure based on amplification of *hrp*-related fragments was examined for sensitive and specific detection of plant pathogenic xanthomonads associated with pepper and tomato seeds. The complexity of the xanthomonads community found on seeds was assessed, and confirmed previous results obtained by other researchers using different methods (Gitaitis et al., 1987, 1992). Furthermore, the combined sensitivity and specificity of the DNA amplification approach certainly makes the procedure an indispensable tool for the detection and identification of plant pathogenic xanthomonads in seeds and other propagative materials. Although the different xanthomonads could not be distinguished by the size of the amplified DNA fragment, the restriction fragment length polymorphism present in the DNA sequences related to the *hrp* genes obtained from different strains of *Xanthomonas* was valuable for differentiation of these plant pathogenic xanthomonads. Furthermore, the comparison of the restriction profile to predetermined profiles of several pathovars of *X. campestris* (Chapter 4) allowed the tentative and precise identification of these plant pathogenic xanthomonads.

CHAPTER 8

SUMMARY AND CONCLUSIONS

The plant pathogenic xanthomonads form a diverse group of usually yellow-pigmented bacteria that occur worldwide and cause disease on many plants. The differentiation of these bacteria at the subgeneric level is by the capability of the bacterial strain to cause characteristic disease or by reference to their host range (Dye et al., 1980, Vauterin et al., 1990a, Young et al., 1992). In fact, it is almost impossible to differentiate the xanthomonads by biochemical and physiological features without knowing their plant hosts (Bradbury, 1984, Dye, 1962, Holt et al., 1994, Schaad, 1988, Van den Mooter and Swings, 1990). On the contrary, the genomic relationship among plant pathogenic xanthomonads has revealed a group of bacteria with diverse genetic background (Hildebrand et al., 1990, Palleroni et al., 1993, Vauterin et al., 1993). In this work, I investigated a specific region of the bacterial genome, the DNA sequences related to the hypersensitive reaction and pathogenicity (*hrp*) genes, for differentiation of the xanthomonads and also for the establishment of the evolutionary relationships among them.

Three sets of oligonucleotide primers selected from the DNA sequence of the *hrp* genes of *X. campestris* pv. vesicatoria were tested for amplification of DNA fragments from different plant pathogenic xanthomonads. Two sets of primers, RST2 plus RST3 and RST9 plus RST10, are specific for the *hrpB*, and the set RST21 plus RST22 is specific for a region of the *hrpC/D* of *X. campestris* pv. vesicatoria 75-3. DNA fragments were amplified from *X. fragariae* and from 31 of 33 plant pathogenic taxa of *X. campestris* tested, which comprise at least 28 different pathovars of this species. In all cases, each set of primers amplified DNA fragments identical in size, suggesting a high degree of structural conservation between operon, as seen with the primers RST21 and RST22. Furthermore, the pathogenicity of several nonpathogenic Tn*3*-*gus* mutants of *X. campestris* pv. vesicatoria 85-10 was fully restored with cloned regions of DNA of *X. campestris* pv. vesicatoria group B and *X. campestris* pv. pelargonii, from which the *hrp*-related fragments were amplified. This supports the contention that the fragments were amplified from DNA sequences which also control the pathogenicity in other xanthomonads.

In contrast to the narrow spectrum of oligonucleotide primers previously described for detection and identification of only certain strains of *X. campestris* (Garde and Bender, 1991; Hartung, 1992), the *hrp*-specific primer sets RST2 plus RST3 and RST21 plus RST22 were useful for identification of a large number of plant pathogenic xanthomonads. This is not surprising, because the *hrp* region seems to be very conserved among different plant pathogenic xanthomonads as determined in the present and previous studies (Bonas et al., 1991; Stall and Minsavage, 1990). On the other hand, the primers RST9 and RST10 allowed amplification of DNA fragment only from a limited number of pathovars of *X. campestris*, although the *hrp* fragment amplified with these primers from *X. campestris* pv. vesicatoria 75-3 hybridized to the majority of the plant pathogenic xanthomonads included in this study. These results indicate differences in the DNA sequences of the xanthomonads corresponding to one or both primers used. However, the set of primers RST9 and RST10 seems useful for specific detection of strains of *X. campestris* pv. vesicatoria group A, *X. campestris* pv. fici, *X. campestris* pv. physalidicola, and *X. campestris* X198.

Although no size variation was observed for the *hrp*-related fragments amplified from different plant pathogenic xanthomonads, the restriction analysis of the

840- and 1,075-bp DNA fragments with frequent-cutting endonucleases indicated the presence of characteristic sequence variation. A study carried out to determine the extension of the diversity of *hrp*-related sequences of 192 strains of plant pathogenic xanthomonads revealed that only a few taxa of the xanthomonads are homogeneous. This includes *X. fragariae*, *X. campestris* pv. begoniae, *X. campestris* pv. campestris, *X. campestris* pv. malvacearum, and *X. campestris* pv. pelargonii. The homogeneous population structure of these taxa has also been supported by using different methods, such as DNA-DNA hybridization, fatty acid composition, and SDS-PAGE of proteins (Vauterin et al., 1990a, 1991a; Yang et al., 1993). On the contrary, the majority of the pathovars of *X. campestris* seem to comprise an heterogeneous group of strains as determined by the analysis of the *hrp*-related sequences. Whereas strains of some pathovars formed distinct and uniform subgroups, e.g. *X. campestris* pv. citri, *X. campestris* pv. dieffenbachiae, and *X. campestris* pv. vesicatoria, other pathovars were composed of a highly diverse group of strains. The latter may be well characterized by the population structure determined for *X. campestris* pv. citrumelo. The 16 strains of *X. campestris* pv. citrumelo included in the study were separated in nine different groups on the basis of the restriction banding profiles generated for the two *hrp*-related fragments digested with four different endonucleases. The diverse nature of the strains of *X. campestris* pv. citrumelo has been reported previously based on DNA homology, restriction fragment length polymorphism analysis, fatty acid composition, and SDS-PAGE of proteins (Egel et al., 1991; Gabriel et al., 1988, 1989; Graham et al., 1990; Hartung and Civerolo, 1987, 1989; Vauterin et al., 1991a).

The most striking feature of the restriction analyses of the amplified *hrp*-related fragments is the possibility for differentiation of almost all the pathovars and groups of *X. campestris* included in the study on the basis of the restriction banding profile of the *hrp*-related sequences. Fifty different restriction fragment length polymorphism groups of plant pathogenic xanthomonads were established based on the banding patterns generated by the analysis of two *hrp*-related fragments digested with four endonucleases. Although the banding pattern for a combination *hrp*-related fragment and endonuclease may be shared by more than one group, the profile of all combinations *hrp* fragments and endonucleases allowed the specific differentiation and identification of the different plant pathogenic xanthomonads included in the study. However, some strains of different groups of plant pathogenic xanthomonads could not be distinguished by restriction analysis of the *hrp*-related sequences. These include a strain of *X. campestris* pv. incanae and one of *X. campestris* pv. carotae which were identical to strains of *X. campestris* pv. campestris; a strain of *X. campestris* pv. phaseoli identical to strains of *X. campestris* pv. glycines group A, strains F59 and F86 of *X. campestris* pv. citrumelo were indistinguishable from strains of *X. campestris* pv. pruni, strain X125 of *X. campestris* pv. fici group A was identical to strains of *X. campestris* pv. poinsettiicola group A, strain XV2 of *X. campestris* pv. vitians was identical to strains of *X. campestris* pv. malvacearum, and strain X52 isolated from *Hibiscus* sp. was indistinguishable from strains of *X. campestris* pv. poinsettiicola group B. No further investigation was carried out with those strains, but it may be necessary a better assessment of the identity of these strains by using different approaches.

To illustrate the usefulness of the amplification and analysis of *hrp*-related fragments in the study of plant pathogenic xanthomonads, I examined the xanthomonads associated with pepper and tomato seeds. A complex xanthomonad community was found in association with these seeds as determined by restriction analysis of *hrp*-related DNA sequences and confirmed by fatty acid analysis. Among the 56 strains isolated from pepper and tomato seeds or seedlings, only nine strains were positively identified as *X. campestris* pv. vesicatoria, the pepper and tomato pathogen. The other strains formed two distinct groups. Seven strains did not produce a reaction on pepper and tomato plants and they failed to produce DNA sequences similar to the *hrp* genes. These strains resemble the opportunistic xanthomonads that have been described previously in association with pepper and tomato transplants (Gitaitis et al., 1987, 1992). The other group of xanthomonads included potential plant pathogenic strains that were not pathogenic to pepper and tomato, but instead caused a hypersensitive reaction or no reaction on these plants under artificial inoculation. These strains were tentatively identified as belonging to different pathovars of *X. campestris*, but not to *X. campestris* pv. vesicatoria, on the basis of the *hrp* analysis and fatty acid composition. The presence of a diverse group of xanthomonads associated with pepper and tomato seeds and transplants is of major significance for the health inspection in certification programs, particularly with the procedures currently available for the detection and identification of plant pathogenic xanthomonads (Holt, 1994; Saettler et al., 1989; Schaad, 1988). Although more investigation may be necessary, the combined specificity and sensitivity of the DNA amplification approach of *hrp*-related fragments seems highly promising for the detection and identification of plant pathogenic xanthomonads associated with propagative plant materials.

Differences observed in the number of common restriction fragments of the amplified DNA sequences related to the *hrp* genes indicated that there is variation in the relatedness of the *hrp* genes of the different strains of plant pathogenic xanthomonads. The relatedness of the *hrp* genes of the different plant pathogenic xanthomonads was further investigated by phylogenetic analysis. This analysis revealed a diverse evolutionary relationship for the *hrp* genes of the plant pathogenic xanthomonads The hypothesis of coevolution of the *hrp* region with the rest of the genome from a common ancestor is supported in several cases. For instance, the similarity in the *hrpB* and *hrpC/D* regions examined in this study for the groups A and B of *X campestris* pv vesicatoria was less than 0 51 This genetic divergence of the *hrp* genes is in agreement with the results obtained when the entire genome of these two groups of *X. campestris* pv vesicatoria were compared on the basis of DNA homology (Stall et al. 1994) Although these pathogens cause similar diseases on solanaceous plants, their *hrp* genes are genetically diverse to the same extent as the rest of the genome Furthermore, these comparisons do not support the contention that there was a higher selective pressure for the *hrp* sequences than for other regions of the genome nor that a horizontal movement of the *hrp* region of the genome may have occurred between strains of the two groups of *X. campestris* pv vesicatoria. Further evidence to support the coevolution of the *hrp* genes and the rest of the genome involves the pathovars *X. campestris* pv carotae, *X. campestris pv* gardneri, and *X campestris* pv pelargonii Although these xanthomonads have very distinct host ranges, they are genetically closely related based on DNA DNA hybridization (Hildebrand et al., 1989, Palleroni et al , 1993) In relation to the *hrp* genes they are not only monophyletic but also very closely related. This example points to a coevolution of the *hrp* genes and the rest of the genome from a common bacterial ancestor for certain plant pathogenic xanthomonads and also substantiates the divergence between *hrp* genes and host speciation An important conclusion from the phylogenetic studies is the divergence between *hrp* genes and host specificity The *hrp* genes are essential for the development of disease on compatible hosts and hypersensitive reaction on both resistant host and nonhost plants (Willis et al., 1991) Previous work has demonstrated the functional conservation of the *hrp* genes and the lack of host speciation (Arlat et al. 1992, Bonas et al. 1991). Although the *hrp* genes are necessary in the plant-pathogen interaction, other factors in the bacterial pathogen are likely to be involved in host specialization (Fenselau et al., 1992, Gough et al., 1992).

On the contrary, there is also indication of horizontal movement of the *hrp* genes among plant pathogenic xanthomonads. This hypothesis is supported by two lines of evidences, the presence of similar *hrp* sequences in strains with divergent genetic background and variability in the relatedness between strains depending on the *hrp* region examined. In the first case, the *hrp* genes of the strains of *X. campestris* pv. vesicatoria A were very closely related to the homologous sequences of the strains of *X. campestris* pv. citrumelo. The estimated genetic relatedness between strains of these two pathovars for both *hrp*-related regions examined ranged in all from 0.71 to 0.96. Furthermore, the phylogenetic analysis strongly supports a monophyletic relationship for the *hrp* genes of the strains of both pathovars. In contrast, DNA homology studies indicated that the strains of these two pathovars are not closely related, with genomic similarity usually below 0.58 (Egel, 1991).

Stronger evidence to support the contention of horizontal movement of the *hrp* genes among the plant pathogenic xanthomonads is the variability in the relatedness between strains for the two distinct *hrp* regions examined. For instance, the *hrpC/D* region of *X. campestris* pv. vignicola is phylogenetically monophyletic and closely related to the homologous *hrp* region of a group of pathovars of *X. campestris* that includes *X. campestris* pv. bilvae, *X. campestris* pv. citri, *X. campestris* pv. glycines A, *X. campestris* pv. malvacearum, *X. campestris* pv. phaseoli B, *X. campestris* pv. phaseoli "fuscans", and *X. campestris* pv. vitians B. On the contrary, the *hrpB* region of *X. campestris* pv. vignicola though monophyletic is closely related to the homologous *hrp* region of *X. campestris* pv begoniae, *X campestris* pv dieffenbachiae, *X. campestris* pv maculifoliigardeniae, *X campestris* pv manihotis, and *X. campestris* pv phaseoli A. Since there is no genetic and functional indication of selection pressure regarding the *hrp* genes, the most likely hypothesis to explain the source of the variability in the relatedness for these two regions of the genome of *X campestris* pv vignicola remains in the origin of the *hrpB* and *hrpC/D* genes from distinct ancestors The coexistence in the same biological niche may have provided opportunities for the lateral movement of this region of bacterial genome between xanthomonads. The horizontal movement of genetic material between bacteria is a common and important mechanism in bacterial evolution (Krawiec and Riley, 1990). Furthermore the resemblance at the protein level of the *hrp* genes of the xanthomonads with genes involved in the secretion of pathogenicity factors of genetically distant organisms such as the animal pathogens of the genus *Yersinia* (Fenselau et al 1992, Gough et al., 1992), is also intriguing If this means a convergent functional evolution or a common bacterial ancestor remains to be clarified. Nevertheless, the phylogeny of the *hrp* genes of plant pathogenic xanthomonads may provide a framework and a rational basis through which origins and differentiation of the xanthomonads may be assessed.

The classification of plant pathogenic bacteria at pathovar level was not based initially on the genetic or other intrinsic characteristic of the organism but rather on the host from which the bacteria were isolated (Bradbury 1984, Dye et al , 1980) Furthermore, comprehensive studies on the genetic and phenotypic characteristics have supported the existence of a high degree of diversity among strains of a given pathovar of *X campestris* The genetic analysis of the *hrp*-related sequences also indicates a large diversity among the plant pathogenic xanthomonads. Furthermore, the diversity or uniformity of the different taxa of xanthomonads assessed on the basis of restriction analysis of *hrp*-related sequences apparently agrees very closely with the structure established by using other methods. However, the restriction banding profiles generated for the *hrp*-related fragments may be an easier and a more discriminating approach for identification of plant pathogenic xanthomonads, compared to other nucleic acid-based approaches, such as the genomic fingerprinting or restriction fragment length polymorphism analysis by using random or specific DNA probes. However, more extensive work is necessary to further characterize the *hrp*-related sequence variation in other groups of plant pathogenic xanthomonads. Furthermore, DNA-DNA hybridization data is also necessary to determine the consistency of the groupings established on such a small region of the bacterial genome as the *hrp* genes

APPENDIX A

BACTERIAL STRAINS AND PLASMIDS USED IN THIS STUDY

Table A-1 List of bacterial strains used in this study and their source

Pathovar	Strain	Source[a]
Xanthomonas		
X. campestris		
pv alfalfae	G 22 (KS)	DLS
	82-1	RES
pv armoraciae	63-27 417 756	JBJ
pv begoniae	XCB9 X274 X281 X329, X610 X627, X1490 X1492 X1496	ARC
pv bilvae	XCB	ELC
pv campestris	33913	ATCC
	62-1, 62-9a, 65-6b 70-3, 70-5 71-2, 83 1, 83 2	JBJ
pv carotae	#3 #5, #7, #9 #12, #13, #16	RES
pv celebensis	6207	ARC
pv citri		DPI
canker A	3213, 3340, 9760-2, 9771, T1 115A	
canker B	B64, B69 B80 B84, B93, B94, B148	
canker C	70C, 338, 339 340, 341,342	
pv citrumelo	F1 F6 F54, F59 F86 F94, F100 F228 F254, F274 F306, F311, F348 F361 F378, 3166	DPI
pv dieffenbachiae	X729	RES

Continued on following page

Table A-1—*Continued*

Pathovar	Strain	Source[a]
pv. dieffenbachiae	X260, X422, X736, X738, X739, X745, X757, X763, X790, X1238, X1241, X1246, X1272, X1292, X1298, X1426	ARC
pv. fici	X125, X151, X208, X212, X224, X548, X687, X702	ARC
pv. gardneri	XG101	DCH
	XV6, 1066	RES
pv. glycines	G-56, 86-16, 86-17, 86-18, 86-20, 87-2	RES
pv. holcicola	G 23	JEL
pv. incanae	9561-1, UK152, UK153	RES
pv. maculifoliigardeniae	X22j	DPI
pv. malvacearum	RIATC	RES
	XcmA, XcmB, XcmC, XcmD, XcmE, XcmF, XcmH, XcmN	DWG
pv. manihotis	Xm125D	RES
pv. papavericola	XP5	DCH
pv. pelargonii	XCP2, XCP10, XCP17, XCP36, XCP44, XCP54, XCP58, XCP60	JBJ
pv. phaseoli	81-19, 82-1, 82-17, 85-6, EK11, XCPH4, XP20	RES
pv. phaseoli "fuscans"	XP163A	DCH
pv. physalidicola	XP172	DCH
pv. poinsettiicola	071-424, X87, X202, X259, X351, X352, X354	RES
pv. pruni	X1219L, X1219S, X1220L, X1220S	ARC
pv. raphani	16B, 69-2, 69-4B, 69-8R	RES
pv. secalis	XC129C	RES
pv. translucens	80-1	RES

Continued on following page

Table A-1—*Continued*

Pathovar	Strain	Source[a]
pv. taraxaci	XT11A	DCH
pv. vesicatoria		RES
group A	75-3, 82-4, 85-10, 87-21, 87-35T, 87-44T, 87-48T, 89-8, 89-10, 90-20, 90-21, 90-27, 90-40, 90-60, 91-66, 91-72, 92-11, 92-15, 92-16, 92-17, 92-119, 1712, 6107, XV14, XV17	
group B	141, 0226A, 0350A, 695, 853, 1062, B-3, B-20, BA28-1, BV5-5, XV56	RES
group C	92-118, 92-120	RES
pv. vignicola	81-30, 82-38, G-55	RES
pv. vitians	ICPB101, ICPB164, XV1T, XV2, XV3, X1215	RES
undetermined and isolated from *Feronia* sp.	XCF	ELC
undetermined and isolated from *Hibiscus* sp.	X10, X27, X52,	DWG
undetermined and isolated from *Strelitzia reginae*	X198	ARC
undetermined and opportunistic	1, 2, 2-8, 4, 7, 8, 9, 10, 11, 12, 17, 661, 663, AAI, CB, Danny Gay, INA, INA42, INA69, RG-1, S52, T-55, T-56, Toad Flax, TP78	RES
X. albilineans	91-065	JCC
X. fragariae	GC-6259, GC-6265, X1238, X1241, X1244, X1246, X1292, X1298, X1426	ARC
X. maltophilia		RES

Continued on the following page

Table A-1—*Continued*

Pathovar	Strain	Source[a]
Acidovorax avenae		
A. avenae subsp. *avenae*	UK142 A	JBJ
A. avenae subsp. *citrulli*	UK20	JBJ
Agrobacterium tumefaciens	LBA1050	BJS
Clavibacter michiganense subsp. *michiganense*	69-1, 75-1	RES
Erwinia		
E. carotovora subsp. *carotovora*	K-SR-347, B-SR38	JAB
E. herbicola	NF-33	RES
E. stewartii	SW2	DLC
Pseudomonas		
P. solanacearum	K60 GMI1000	AK
P. syringae		
pv. syringae	INB	RES
pv. tomato	987	RES
Xylella fastidiosa	89-1	DLH

[a] AK, A. Kelman, University of Wisconsin, Madison, WI, ARC, A. R. Chase, University of Florida, Apopka, FL, ATCC, American Type Culture Collection, Rockville, MD BJS, B. J. Staskawicz, University of California, Berkaly, CA, DCH, D. C. Hildebrand, University of California, Berkely, CA, DLC, D. L. Coplin, Ohio State University, Columbus, OH, DLH, D. L. Hopkins, University of Florida, Leesburg, FL, DLS, D. L. Stuteville, Kansas State University, Manhattan, KS, DPI, Department of Plant Industry, Gainesville, FL, DWG, D. W. Gabriel, University of Florida, Gainesville, FL, ELC, E. L. Civerolo, U.S. Department of Agriculture, Beltsville, MD, JAB, J. A. Bartz, University of Florida, Gainesville, FL, JBJ, J. B. Jones, University of Florida, Bradenton, FL, JCC, J. C. Comstock, U.S. Department of Agriculture, Canal Point, FL, JEH, J. E. Hunter, Cornell University, Geneva, NY, JEL, J. E. Leach, Kansas State University, Manhattan, KS, RES, R. E. Stall, University of Florida, Gainesville, FL.

Table A.2. List of bacterial strains and plasmids used in molecular transformation and conjugation in this study

Strain	Relevant characteristics	Source or reference^a
Bacteria		
Xanthomonas campestris pv. vesicatoria		
22	nonpathogenic Tn3-gus insertion mutant of strain 85-10	Bonas et al., 1991
44	nonpathogenic Tn3-gus insertion mutant of strain 85-10	Bonas et al., 1991
75	nonpathogenic Tn3-gus insertion mutant of strain 85-10	Bonas et al., 1991
85	nonpathogenic Tn3-gus insertion mutant of strain 85-10	Bonas et al., 1991
137	nonpathogenic Tn3-gus insertion mutant of strain 85-10	Bonas et al., 1991
318	nonpathogenic Tn3-gus insertion mutant of strain 85-10	Bonas et al., 1991
Escherichia coli		
DH5α	F⁻recA φ80dlacZM15	BRL
HB101	F⁻recA	BRL
Plasmids		
pLAFR3	Tc^r*rlx*$^+$ RK2replicon	Staskawicz et al., 1997
pBluescript-KS$^+$	Amp^r *Bluescript*	Stratagene
pRK2013	Km^{r}TraRK2$^+$Mob$^+$ColE1 replicon	Figurski and Helinski, 1979
pXV9	pLAFR3 cosmid clone from *X. c.* pv. vesicatoria 75-3	Bonas et al., 1991
pXV847	pBluescript clone containing internal *Sau* 3A *hrp* fragment amplified from *X. c.* pv. vesicatoria 75-3	This study
pXV1111	pBluescript clone containing internal *Sau* 3A *hrp* fragment amplified from *X. c.* pv. vesicatoria 75-3	This study
pXV5.1	pLAFR3 *Eco* RI subclone from pXV9	This study
pXV5.5	pLAFR3 *Eco* RI subclone from pXV9	This study

Continued on the following page

Table A-2—*Continued*

Strain	Relevant characteristics	Source or reference[a]
pXV56/3-48	pLARF3 cosmid clone from *X. c.* pv. vesicatoria XV56	This study
pXCP58/2	pLARF3 cosmid clone from *X. c.* pv. pelargonii	This study

[a] BRL, Bethesda Research Laboratories, Gaithersburg, MD. Stratagene, Stratagene, Inc., La Jolla, CA.

APPENDIX B

SAS PROGRAM TO ESTIMATE THE SIMILARITY VALUES FROM RESTRICTION FRAGMENT DATA

```
options ls=80 ps=54 nodate nonumber;

data dna_type;
 infile bands lrecl=835;
 input bacteria $ band1-band100;   /* Read data from file Bacteria and set number of
*/
                                    /* fragments compared */

proc sort data=dna_type;
 by bacteria;

data dna_type;
 set dna_type;
 bact1=_n_;
 bact2=_n_;

proc transpose data=dna_type out=transout;   /* Create a new set with rows of the */
 var band1-band100;                                 /* original data matrix becoming
*/
                                               /* columns, and columns becoming */
                                               /*  rows */

data coefmtx;
 coef=.;
 bact1=.;
 bact2=.;

 %macro calccoef;                       /* Invoke the macro processor and execute */
  %do i=1 %to 3;                        /* the caulculations until j = # of strains to be */
  %do j=%eval (&i+1) %to 4;             /* compared, i = j - 1 */
```

```
data temp;                          /* Compare fragment data between two */
 set transout (keep=col&i col&j);   /* strains and create a new data set */
 column1=col&i;
 column2=col&j;
 if column1=1 & column2=1 then prod=1
  else prod=0;
 keep column1 column2 prod;

proc means noprint data=temp;
 var prod column1 column2;
 output out=outset sum=prodsum col1sum col2sum;

data outset;                               /* Determine the similarity value */
 set outset;                               /* between two strains */
 coef=(2*prodsum) / (col1sum+col2sum);
 bact1=&i;
 bact2=&j;
 keep coef bact1 bact2;

data coefmtx;
 set coefmtx outset;
%end;
 %end;
%mend calccoef;

%calccoef

data coefmtx;
 set coefmtx;
 if bact1=  then delete;

proc sort data=coefmtx;
 by bact2

data coefmtx;
 merge coefmtx (in=i) dna_type (keep=bact2 bacteria);
 by bact2;
 if i=1;
 bacter2=bacteria;
 drop bacteria;
```

```
proc sort data=coefmtx,                          /* Sort the result by strain name */
 by bact1,

data coefmtx,
 merge coefmtx (in=i) dat_type (keep=bact1 bacteria),
 by bact1
 if i=1,
 bacter1=bacteria,
 drop bacteria,
 label bacter1="Strain" bacter2="Strain"
   coef="strain",

proc tabulate f=9.4 formchar=          ,                /* Print out the result */
 title "Similarity values for bacteria",
 class bacter1 bacter2
 var coef;
 table bacter1,bacter2*coef;
 keylabel sum=" "

 run,
```

APPENDIX C

SAS PROGRAM TO ESTIMATE THE NUCLEOTIDE SUBSTITUTION BASED ON RESTRICTION FRAGMENT DATA

```
data frag
  infile fragment               /* Read data of similarity values from */
  input similar @@              /* file fragment */

g0=similar**0.25,                  /* Determine the probability that */
   g=(similar*(3-(2*g0)))**0.25;   /* a restriction site has remained */
   g1=G0                           /* unaltered */
   x=1,
   do while (g1 ne g);
     g1=g
     if x ne 50 then do,
     g=(similar*(3-(2*g1)))**0.25,
     x=x+1,
     end,
     end,
  do differe=-((2/r)*(log(g)))     /* Determine the nucleotide substitution, */
     end,                          /* r is the number of nucleotides in the */
     output,                       /* recognition site of the restriction */
                                   /* endonuclease */

proc print split='*'               /* Print out the result */
  var similar g0 g differe x,
  label similar='Similarity'
     g0='Initial*probability'
     g='Final*probability'
     differe='Nucleotide*substitution'
     x='Number*iteration'
  title 'DNA similarity and nucleotide substitution',
  title2 'Nei and Li (1979) procedure',

run
```

APPENDIX D

ESTIMATES OF SIMILARITY VALUES FOR PLANT PATHOGENIC XANTHOMONADS BASED ON COMPARISON OF RESTRICTION FRAGMENT DATA OF THE *hrp* RELATED DNA SEQUENCES

Table D-1. Similarity values for plant pathogenic strains of different pathovars of *Xanthomonas campestris* and *X. fragariae* based on the genetic analysis of the *hrpC/D* related DNA sequences

Species/Pathovar	Species/Pathovar									
	2	3	4	5	6	7	8	9	10	11
1 pv alfalfae	9583	3182	5000	5217	5217	7083	3182	3636	3182	5833
2 pv alfalfae		3182	5000	5217	5217	6667	.3182	3636	3182	5833
3 pv armoraciae			2500	3333	3810	3182	1 000	8500	1 000	3636
4 pv begonia A				9048	8571	3636	.2500	3000	.2500	3636
5 pv begonia B					9545	3913	3333	.3333	3333	3913
6 pv begoniae C						3913	3810	3810	3810	.3913
7 pv bilvae							.3182	4091	3182	7917
8 pv campestris								8500	1 000	3636
9 pv carotae A									8500	4545
10 pv carotae B										3636
11 pv citri A										

Continued on following page

Table D-1—*Continued*

Species/Pathovar	Species/Pathovar									
	12	13	14	15	16	17	18	19	20	21
1 pv alfalfae	5000	5000	8163	9388	8333	8333	.9167	8511	7083	8333
2 pv alfalfae	.5000	5000	7755	8980	7917	7917	8750	8936	6667	7917
3 pv armoraciae	3182	3182	.3111	3111	2727	2727	2727	2791	.2727	.3182
4 pv begoniae A	1818	1818	.3556	4889	5455	5455	5909	6047	5000	.5455
5 pv begoniae A	.2174	2174	3830	5532	5217	5217	5652	5778	4783	6087
6 pv begonia B	2609	2609	4255	5532	5217	5217	5652	5778	5217	6522
7 pv bilvae	7083	7083	6531	7347	6667	6667	7083	.6383	6250	6667
8 pv campestris	3182	3182	3111	.3111	.2727	.2727	2727	2791	2727	3182
9 pv carotae A	4091	4091	3556	3556	3182	.3182	3182	3256	3182	3636
10 pv carotae B	3182	3182	3111	3111	2727	.2727	2727	.2791	2727	3182
11 pv citri A	7500	.7500	4898	6122	5833	5833	5833	5957	4583	5833
12 pv citri B		1 000	5306	5306	4583	4583	4583	4255	5833	5000
13 pv citri C			5306	5306	4583	4583	4583	4255	.5833	5000
14 pv citrumelo				7600	7755	7755	7347	6667	6939	6939
15 pv citrumelo					8163	8163	8980	8333	6939	8980
16 pv citrumelo						1 000	9167	8936	7917	7500
17 pv citrumelo							9167	8936	7917	7500
18 pv citrumelo								9362	7917	7917
19 pv citrumelo									7234	7660
20 pv citrumelo										7500
21 pv citrumelo										

Continued on following page

Table D-1—*Continued*

Species/Pathovar	Species/Pathovar									
	22	23	24	25	26	27	28	29	30	31
1 pv alfalfae	8750	.4167	4167	6818	9167	7755	7755	.2727	3636	5833
2 pv alfalfae	8333	4167	4167	6818	8750	7347	7347	2727	3636	5833
3 pv armoraceae	2727	1818	1818	2500	2727	3556	.3556	4000	.9000	3636
4 pv begoniae A	5455	.6364	6818	7500	5909	3556	.3556	2000	.3500	3636
5 pv begoniae A	5652	6957	7391	7143	5652	3830	3830	2381	.3333	3913
6 pv begonia B	5652	6522	6957	7143	5652	4255	4255	2857	.3810	3913
7 pv bilvae	6667	.3333	.3333	5000	7083	6939	6939	3636	.3182	7917
8 pv campestris	2727	1818	1818	2500	2727	3556	3556	4000	9000	3636
9 pv carotae A	3182	.2273	.2273	3000	3182	4000	4000	4500	8500	4545
10 pv carotae B	2727	1818	1818	2500	.2727	3556	3556	4000	9000	.3636
11 pv citri A	5417	2917	.2500	5000	5833	5306	5306	2273	3636	1 000
12 pv citri B	4583	2500	.2083	3182	4583	6939	6939	3182	3182	7500
13 pv citri C	4583	2500	2083	3182	4583	6939	.6939	3182	3182	7500
14 pv citrumelo	8163	4898	4898	4889	7347	8400	8400	3111	3556	4898
15 pv citrumelo	8163	4490	4490	6667	8980	8000	8000	2667	3556	6122
16 pv citrumelo	7083	4167	4167	6818	9167	7347	7347	.2273	3636	5833
17 pv citrumelo	7083	4167	4167	6818	9167	7347	7347	2273	3636	5833
18 pv citrumelo	7917	4583	4583	7273	1.000	7347	7347	2273	.3636	5833
19 pv citrumelo	7234	4255	4255	7442	9362	6667	6667	2326	.3721	5957
20 pv citrumelo	7083	4167	4583	.6364	.7917	8163	8163	2727	3636	4583
21 pv citrumelo	8333	4167	4583	7273	7917	7347	7347	3182	3636	.5833
22 pv citrumelo		.5000	5417	6818	7917	7347	7347	.2727	3182	.5417

Continued on following page

Table D-1—*Continued*

Species/Pathovar	Species/Pathovar									
	22	23	24	25	26	27	28	29	30	31
23 pv. dieffenbachiae A			9583	5000	.4583	4082	4082	1364	.2273	2917
24 pv. dieffenbachiae A				5455	4583	4082	4082	1818	.2273	2500
25 pv. dieffenbachiae B					7273	4889	4889	.2000	3500	.5000
26 pv. fici A						7347	7347	.2273	3636	.5833
27 pv. fici A							1.000	.3111	4000	.5306
28 pv. fici A								.3111	4000	.5306
29 pv. fici B									4000	.2273
30 pv. gardneri										.3636
31 pv. glycines A										

Continued on following page

Table D-1—*Continued*

Species/Pathovar	Species/Pathovar									
	32	33	34	35	36	37	38	39	40	41
1 pv alfalfae	.2727	1667	3182	5217	4889	.5306	4000	3556	4681	5833
2 pv alfalfae	2727	1667	3182	5217	4889	.5306	4000	3556	4681	5833
3 pv armoraciae	4000	3182	1 000	3333	3415	2667	8780	9268	1860	3636
4 pv begoniae A	2000	1818	2500	8095	3415	.8444	3415	2927	7442	3636
5 pv begoniae A	2381	1739	3333	9091	3721	8936	4186	3721	8000	3913
6 pv begoniae B	.2857	.2174	3810	9091	3721	8511	.4651	4186	7556	3913
7 pv bilvae	.3636	1250	3182	4348	7111	.4082	4000	4000	3830	7917
8 pv campestris	4000	.3182	1 000	.3333	3415	.2667	8780	9268	1860	3636
9 pv carotae A	4500	.3182	8500	.3333	4390	3111	.8780	9268	.2326	4545
10 pv carotae B	4000	3182	1 000	.3333	3415	.2667	8780	9268	1860	.3636
11 pv citri A	2273	.2500	3636	3478	8000	4082	4444	4444	2979	1 000
12 pv citri B	3182	1667	3182	2609	6667	2449	4000	4000	2128	7500
13 pv citri C	3182	1667	3182	2609	6667	2449	4000	4000	2128	7500
14 pv citrumelo	3111	1633	3111	4255	4348	4800	.3478	.3478	5417	4898
15 pv citrumelo	2667	1633	3111	5532	5217	5600	3913	3478	5000	6122
16 pv citrumelo	2273	1667	2727	4783	4444	5306	3111	3111	4681	5833
17 pv citrumelo	.2273	1667	2727	4783	4444	5306	.3111	.3111	4681	5833
18 pv citrumelo	.2273	1667	2727	5652	4889	5714	3556	3111	5106	5833
19 pv citrumelo	.2326	1702	2791	5333	4545	5833	3636	.3182	4783	5957
20 pv citrumelo	.2727	1667	2727	5217	4000	4898	3111	.3111	4681	4583
21 pv citrumelo	3182	2083	3182	5652	4444	6122	4000	.3556	.5106	5833
22. pv citrumelo	.2727	1667	2727	5652	4444	6531	3556	3111	.5957	.5417

Continued on following page

Table D-1—*Continued*

Species/Pathovar	Species/Pathovar									
	32	33	34	35	36	37	38	39	40	41
23 pv. dieffenbachiae A	1364	1250	1818	7391	3111	8163	2667	2222	8936	2917
24 pv. dieffenbachiae A	1818	1250	1818	7826	2667	8571	2667	2222	9362	2500
25 pv. dieffenbachiae B	2000	2273	2500	6667	3415	7111	3415	2927	6047	5000
26 pv. fici A	2273	1667	2727	5652	4889	5714	3556	3111	5106	5833
27 pv. fici A	3111	1633	3556	4255	4783	4400	3913	3913	4167	5306
28 pv. fici A	3111	1633	3556	4255	4783	4400	3913	3913	4167	5306
29 pv. fici B	1 000	1818	4000	2381	2439	2222	3902	4390	1860	2273
30 pv. gardneri	4000	3182	9000	3333	3415	3111	8293	8780	2326	3636
31 pv. glycines A	2273	2500	3636	3478	8000	4082	4444	4444	2979	1 000
32 pv. glycines B		1818	4000	2381	2439	2222	3902	4390	1860	2273
33 pv. holcicola			3182	1304	1778	1633	3111	3111	1277	2500
34 pv. incanae				3333	3415	2667	8780	9268	1860	3636
35 pv. maculifoliigardeniae					4186	8085	4186	3721	8000	3478
36 pv. malvacearum						3478	4286	4286	3182	8000
37 pv. manihotis							3478	3043	8750	4082
38 pv. papavericola								9524	2727	4444
39 pv. pelargonii									2273	4444
40 pv. phaseoli A										2979
41 pv. phaseoli B										

Continued on following page

Table D-1—*Continued*

Species/Pathovar	42	44	45	46	47	48	49	50	51	52
1 pv alfalfae	5217	3256	9167	9388	4091	4091	3636	7917	7660	7660
2 pv alfalfae	5217	3256	8750	8980	4091	4091	3636	7500	7234	7234
3 pv armoraceae	.2381	4615	.2727	3111	4500	4500	8500	.2273	2326	2326
4 pv begoniae A	.2381	2564	.5909	4889	3500	.3500	3500	5000	5116	5116
5 pv begoniae A	.2727	2927	.5652	5532	3810	.3810	3810	4783	5333	5333
6 pv begonia B	.2727	3415	5652	5532	3810	.3810	4286	.4783	.5333	5333
7 pv bilvae	6957	3721	7083	7347	4091	4091	3182	6250	6383	6383
8 pv campestris	2381	4615	2727	3111	4500	4500	8500	.2273	.2326	2326
9 pv carotae A	3333	5128	3182	3556	5000	.5000	8000	2727	.2791	2791
10 pv carotae B	2381	4615	2727	3111	4500	4500	8500	2273	.2326	.2326
11 pv citri A	8261	2791	5833	6122	4091	4091	3636	5417	.5532	5532
12 pv citri B	8261	3256	4583	5306	3636	3636	3182	4583	4681	4681
13 pv citri C	.8261	3256	4583	5306	3636	3636	3182	4583	4681	4681
14 pv citrumelo	5106	3182	7347	7600	3556	3556	3556	7755	7500	7500
15 pv citrumelo	5532	3182	8980	1 000	4000	4000	3556	7755	7500	7500
16 pv citrumelo	5652	.2791	9167	8163	3182	3182	3636	9583	8511	8511
17 pv citrumelo	.5652	.2791	9167	8163	.3182	3182	3636	9583	8511	8511
18 pv citrumelo	4783	.2791	1 000	8980	.3636	3636	.3636	8750	7660	7660
19 pv citrumelo	4889	2857	9362	8333	3256	3256	.3721	8511	7391	7391
20 pv citrumelo	3913	.3256	7917	6939	3636	3636	.3636	7500	7660	7660
21 pv citrumelo	5217	3721	7917	8980	3636	3636	3636	7083	8085	8085
22 pv citrumelo	4783	3256	7917	8163	4091	4091	3182	7083	8085	8085

Continued on following page

Table D-1—*Continued*

Species/Pathovar	Species/Pathovar									
	42	44	45	46	47	48	49	50	51	52
23 pv dieffenbachiae A	2609	1395	4583	4490	2727	2727	2273	4167	4255	4255
24 pv dieffenbachiae A	2174	1860	4583	4490	3182	3182	2273	4167	4681	4681
25 pv dieffenbachiae B	3810	2564	7273	6667	4000	4000	3500	6364	6977	6977
26 pv fici A	4783	2791	1 000	8980	3636	3636	3636	8750	7660	7660
27 pv fici A	5106	3636	7347	8000	4000	4000	4000	7347	7083	7083
28 pv fici A	5106	3636	7347	8000	4000	4000	4000	7347	7083	7083
29 pv fici B	2381	7692	2273	2667	4000	4000	4000	2273	2791	2791
30 pv gardneri	2381	4615	3636	3556	4500	4500	9000	3182	2791	2791
31 pv glycines A	8261	2791	5833	6122	4091	4091	3636	5417	5532	5532
32. pv glycines B	2381	7692	2273	2667	4000	4000	4000	2273	2791	2791
33 pv holcicola	1739	2326	1667	1633	2273	2273	2727	1250	1277	1277
34 pv incanae	2381	4615	2727	3111	4500	4500	8500	2273	2326	2326
35 pv maculifoliigardeniae	2273	2927	5652	5532	4286	4286	3810	4348	4889	4889
36 pv malvacearum	6512	2500	4889	5217	3902	3902	3415	4000	4091	4091
37 pv manihotis	2979	2727	5714	5600	3556	3556	3111	5306	5833	5833
38 pv papavericola	3256	5000	3556	3913	4878	4878	7805	2667	2727	2727
39 pv pelargonii	3256	5000	3111	3478	4878	4878	8293	2667	2727	2727
40 pv phaseoli A	2667	1905	5106	5000	3256	3256	2326	4681	5217	5217
41 pv phaseoli B	8261	2791	5833	6122	4091	4091	3636	5417	5532	5532
42 pv phaseoli "fuscans"		2439	4783	5532	2857	2857	2381	5652	5778	5778
44 pv poinsettiicola B			2791	3182	5128	5128	4615	2791	3333	3333

Continued on following page

Table D-1—*Continued*

Species/Pathovar	Species/Pathovar									
	42	44	45	46	47	48	49	50	51	52
45 pv poinsettiicola A				8980	3636	3636	3636	8750	7660	7660
46 pv pruni					4000	4000	3556	7755	7500	7500
47 pv raphani A						1.000	4500	.2727	3256	3256
48 pv raphani B							4500	.2727	3256	3256
49 pv taraxaci								.3182	2791	2791
50 pv vesicatoria A									8936	8936
51 pv vesicatoria A										1 000
52 pv vesicatoria A										

Continued on following page

Table D-1—*Continued*

Species/Pathovar	Species/Pathovar								
	53	54	55	56	57	58	59	60	61
1 pv alfalfae	4583	5000	4898	4889	3636	9167	7083	.3256	2083
2 pv alfalfae	4583	5000	4898	4889	3636	8750	6667	3256	2083
3 pv armoraceae	.5000	3182	2667	3415	8500	2727	3182	4615	4545
4 pv begoniae A	4091	1818	8000	3415	3500	5909	3636	2564	0909
5 pv begoniae A	4348	2174	8511	3721	3333	5652	3913	2927	1739
6 pv begoniae B	4348	2609	8085	3721	3810	5652	3913	3415	2174
7 pv bilvae	4167	7083	4082	7111	3636	7083	9583	3721	2500
8 pv campestris	5000	3182	2667	3415	8500	2727	3182	4615	4545
9 pv carotae A	5909	4091	3111	4390	9000	3182	4091	5128	3636
10 pv carotae B	5000	3182	2667	3415	8500	.2727	3182	4615	4545
11 pv citri A	5000	7500	4082	8000	4091	5833	8333	.2791	.2917
12 pv citri B	4167	1 000	.2857	6667	3636	4583	7083	.3256	.3333
13 pv citri C	4167	1 000	.2857	6667	3636	4583	7083	3256	.3333
14 pv citrumelo	3265	5306	.5600	4348	3556	7347	6531	3182	.2449
15 pv citrumelo	4490	5306	.5200	5217	3556	8980	7347	3182	2041
16 pv citrumelo	3750	4583	6122	4444	3636	9167	6667	2791	1667
17 pv citrumelo	3750	4583	6122	4444	3636	9167	6667	2791	1667
18 pv citrumelo	4167	4583	5306	4889	3636	1 000	7083	2791	1667
19 pv citrumelo	4255	4255	5417	4545	3721	9362	6383	2857	1702
20 pv citrumelo	3333	5833	5306	4000	3636	7917	6250	3256	2083
21 pv citrumelo	4583	5000	5714	4444	3636	7917	6667	3721	.2500
22 pv citrumelo	4167	4583	6122	4444	3182	7917	6667	3256	2083

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Table D-1—*Continued*

Species/Pathovar	Species/Pathovar								
	53	54	55	56	57	58	59	60	61
23 pv dieffenbachiae A	.2500	2500	7755	.3111	.2273	4583	3333	1395	0833
24 pv dieffenbachiae A	.2917	2083	8163	.2667	2273	4583	3333	1860	0833
25 pv dieffenbachiae B	4545	3182	6667	3415	3500	7273	5000	2564	1364
26 pv fici A	4167	4583	5306	4889	3636	1 000	7083	2791	1667
27 pv fici A	3265	6939	4800	4783	4000	7347	6939	3636	2449
28 pv fici A	3265	6939	4800	4783	4000	7347	6939	3636	2449
29 pv fici B	.3182	3182	2222	2439	4000	2273	.3182	7692	3636
30 pv gardneri	5455	3182	3111	3415	9500	3636	.3182	4615	3636
31 pv glycines A	.5000	7500	4082	8000	4091	5833	8333	.2791	.2917
32 pv glycines B	.3182	.3182	2222	2439	4000	2273	3182	7692	.3636
33 pv holcicola	.3750	1667	1633	1778	3182	1667	1667	2326	.2083
34 pv incanae	.5000	.3182	2667	3415	8500	.2727	3182	4615	4545
35 pv maculifoliigardeniae	4348	.2609	7660	4186	.3333	.5652	4348	2927	1739
36 pv malvacearum	4444	6667	3478	1 000	.3902	4889	7556	2500	.2667
37 pv manihotis	4082	2449	9200	3478	3111	.5714	4082	2727	1224
38 pv papavericola	6222	4000	3043	4286	8780	.3556	4000	5000	4000
39 pv pelargonii	5778	4000	3043	4286	9268	.3111	4000	5000	4444
40 pv phaseoli A	3404	2128	8750	3182	2326	5106	3830	1905	0851
41 pv phaseoli B	5000	7500	4082	8000	4091	5833	8333	.2791	2917
42 pv phaseoli "fuscans"	4348	8261	3830	6512	2857	4783	6957	.2439	.2609
44 pv poinsettiicola B	4186	3256	.2727	.2500	4615	2791	3256	1 000	3256
45 pv poinsettiicola A	4167	4583	5306	4889	.3636	1 000	7083	2791	1667

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Table D-1—*Continued*

Species/Pathovar	53	54	55	56	57	58	59	60	61
46 pv pruni	4490	5306	5200	5217	.3556	8980	7347	3182	2041
47 pv raphani A	4091	3636	3556	.3902	4500	3636	4545	.5128	3182
48 pv raphani B	4091	.3636	3556	.3902	4500	3636	4545	5128	3182
49 pv taraxaci	5000	.3182	3111	.3415	8500	3636	.3182	4615	3636
50 pv vesicatoria A	.3333	4583	6122	4000	3182	8750	6250	.2791	1667
51 pv vesicatoria A	.3830	4681	6667	4091	2791	7660	.6383	.3333	2128
52 pv vesicatoria A	3830	4681	6667	4091	2791	7660	6383	3333	2128
53 pv vesicatoria B		4167	3673	4444	5909	4167	4167	4186	2500
54 pv vignicola			2857	6667	.3636	4583	7083	3256	3333
55 pv vitians A				3478	3111	5306	4082	2727	1224
56 pv vitians B					3902	4889	7556	2500	.2667
57 pv vitians C						3636	3636	4615	3636
58 *X campestris* X198							7083	.2791	1667
59 *X campestris* XCF								3256	2500
60 *X campestris* X52									.3256
61 *X fragariae*									

Table D-2. Similarity values for plant pathogenic strains of different pathovars of *Xanthomonas campestris* and *X fragariae* based on the genetic analysis of the *hrpB* related DNA sequences.

Species/Pathovar	Species/Pathovar									
	2	3	4	5	6	7	8	11	12	13
1 pv alfalfae	1 000	4091	6341	6341	6341	4889	4545	4091	4091	4091
2 pv alfalfae		4091	6341	6341	6341	4889	4545	4091	4091	4091
3 pv armoraciae			4878	4878	4878	5778	9545	5455	5455	5455
4 pv begoniae A				1 000	1 000	6190	5366	5366	5854	5854
5 pv begoniae A					1 000	6190	5366	5366	5854	5854
6 pv begoniae B						6190	5366	5366	5854	5854
7 pv holcicola							.5778	8444	9333	9333
8 pv campestris								5455	5455	.5455
11 pv citri A									8182	8182
12 pv citri B										1 000
13 pv citri C										

Continued on following page

Table D-2—*Continued*

Species/Pathovar	Species/Pathovar									
	14	15	16	17	18	19	20	21	22	23
1 pv alfalfae	9333	8696	8696	9333	9333	9545	8696	1 000	8261	6190
2 pv alfalfae	9333	8696	8696	9333	9333	9545	8696	1 000	8261	6190
3 pv armoraciae	4444	5217	5217	4889	4444	4091	5217	4091	5217	5238
4 pv begoniae A	6667	7442	7442	7143	6667	6341	7442	6341	7442	9231
5 pv begoniae A	6667	7442	7442	7143	6667	6341	7442	6341	7442	9231
6. pv begoniae B	6667	7442	7442	7143	6667	6341	7442	6341	7442	9231
7 pv bilvae	5217	5957	5957	5652	5217	4889	5957	4889	5957	6047
8 pv campestris	4889	5652	5652	5333	4889	4545	5652	4545	5652	5714
11 pv citri A	4444	5217	5217	4889	4444	4091	5217	4091	5217	5714
12 pv citri B	4444	5217	5217	4889	4444	4091	5217	4091	5217	5714
13 pv citri C	4444	5217	5217	4889	4444	4091	5217	4091	5217	5714
14 pv citrumelo		9362	9362	8696	1 000	8889	9362	9333	8936	6512
15 pv citrumelo			1 000	9362	9362	8261	1 000	8696	9583	7273
16 pv citrumelo				9362	9362	8261	1 000	8696	9583	7273
17 pv citrumelo					8696	8889	9362	9333	8936	6977
18 pv citrumelo						8889	9362	9333	8936	6512
19 pv citrumelo							8261	9545	8696	6190
20 pv citrumelo								8696	9583	7273
21 pv citrumelo									8261	6190
22 pv citrumelo										7273
23 pv dieffenbachiae A										

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Table D.2—*Continued*

Species/Pathovar	Species/Pathovar								
	24	25	26	27	28	29	31	34	35	36
1 pv alfalfae	6190	7111	8696	9333	9333	3902	4545	4545	5854	4651
2 pv alfalfae	6190	7111	8696	9333	9333	3902	4545	4545	5854	4651
3 pv armoraciae	5238	5778	5217	4444	4889	6341	5455	9545	5366	5116
4 pv begoniae A	9231	5714	7442	6667	7143	5789	5366	5366	8421	5500
5 pv begoniae A	9231	5714	7442	6667	7143	5789	5366	5366	8421	5500
6. pv begoniae B	9231	5714	7442	6667	7143	5789	5366	5366	8421	5500
7 pv bilvae	6047	5217	5957	5217	5652	4286	8444	5778	6190	8182
8 pv campestris	5714	5333	5652	4889	5333	6829	5455	1 000	5854	5116
11 pv citri A	5714	4889	5217	4444	4889	4390	9091	5455	5854	8837
12 pv citri B	5714	4889	5217	4444	4889	3902	8182	5455	5854	7907
13 pv citri C	5714	4889	5217	4444	4889	3902	8182	5455	5854	7907
14 pv citrumelo	6512	7826	9362	1 000	8696	4286	4889	4889	6190	5000
15 pv citrumelo	7273	8511	1 000	9362	9362	5116	5652	5652	6977	5778
16 pv citrumelo	7273	8511	1 000	9362	9362	5116	5652	5652	6977	5778
17 pv citrumelo	6977	7826	9362	8696	1 000	4762	5333	5333	6667	5455
18 pv citrumelo	6512	7826	9362	1 000	8696	4286	4889	4889	6190	5000
19 pv citrumelo	6190	6667	8261	8889	8889	4390	4545	4545	5854	4186
20 pv citrumelo	7273	8511	1 000	9362	9362	5116	5652	5652	6977	5778
21 pv citrumelo	6190	7111	8696	9333	9333	3902	4545	4545	5854	4651
22 pv citrumelo	7273	8085	9583	8936	8936	5581	5652	5652	6977	5333

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Table D-2—*Continued*

Species/Pathovar	Species/Pathovar									
	24	25	26	27	28	29	31	34	35	36
23 pv. dieffenbachiae A	1.000	6047	7273	6512	6977	6154	5714	5714	9231	.5854
24 pv. dieffenbachiae A		6047	7273	6512	6977	6154	5714	5714	9231	.5854
25 pv. dieffenbachiae B			8511	7826	7826	4286	.5333	5333	6190	.5455
26 pv. fici A				9362	9362	5116	5652	.5652	6977	.5778
27 pv. fici A					8696	4286	4889	4889	6190	5000
28 pv. fici A						4762	.5333	.5333	6667	5455
29 pv. fici B							4390	6829	5769	4500
31 pv. glycines A								.5455	5854	9302
34 pv. incanae									.5854	5116
35 pv. maculifoliigardeniae										6000
36 pv. malvacearum										

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Table D-2—*Continued*

Species/Pathovar	Species/Pathovar									
	37	40	41	42	43	44	45	46	47	48
1 pv alfalfae	5854	6190	4545	4444	8696	4000	8696	8696	4545	4091
2 pv alfalfae	5854	.6190	4545	4444	8696	4000	8696	8696	4545	4091
3 pv armoraciae	4878	.5238	.5455	5333	.5217	5500	5217	5217	9545	1 000
4 pv begoniae A	8947	9231	5366	6667	7442	.5405	7442	7442	.5366	4878
5 pv begoniae A	8947	9231	5366	6667	7442	5405	7442	7442	.5366	4878
6 pv begoniae B	8947	9231	.5366	6667	7442	5405	7442	7442	.5366	4878
7 pv balvae	.5714	6047	8444	9565	.5957	3902	5957	5957	5778	.5778
8 pv campestris	.5366	.5714	5455	5333	5652	6000	5652	5652	1 000	9545
11 pv citri A	5366	5714	9091	8000	5217	4000	5217	5217	5455	5455
12. pv citri B	.5854	.5714	8182	9333	5217	3500	5217	5217	5455	5455
13 pv citri C	5854	5714	8182	9333	.5217	3500	5217	5217	5455	5455
14 pv citrumelo	6190	6512	4889	4783	9362	4390	9362	9362	4889	4444
15 pv citrumelo	.6977	7273	.5652	5532	1 000	4762	1 000	1 000	5652	5217
16 pv citrumelo	6977	7273	5652	5532	1 000	4762	1 000	1 000	5652	5217
17 pv citrumelo	6667	6977	.5333	5217	9362	4390	9362	9362	5333	4889
18 pv citrumelo	6190	6512	4889	4783	9362	4390	9362	9362	4889	4444
19 pv citrumelo	5854	.6190	4545	4444	8261	4500	8261	8261	4545	4091
20 pv citrumelo	6977	7273	5652	5532	1 000	4762	1 000	1 000	5652	5217
21 pv citrumelo	5854	6190	4545	4444	8696	4000	8696	8696	4545	4091
22 pv citrumelo	.6977	.7273	.5652	5532	9583	5238	9583	9583	5652	5217
23 pv dieffenbachiae A	9231	1 000	5714	6047	7273	5789	7273	7273	.5714	5238
24 pv dieffenbachiae A	9231	1 000	5714	6047	7273	5789	7273	7273	.5714	5238

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Table D-2—*Continued*

Species/Pathovar	Species/Pathovar									
	37	40	41	42	43	44	45	46	47	48
25 pv dieffenbachiae B	5714	6047	5333	4783	8511	3415	8511	8511	5333	5778
26 pv fici A	6977	7273	5652	5532	1 000	4762	1 000	1 000	5652	5217
27 pv fici A	6190	.6512	4889	4783	9362	4390	9362	9362	4889	4444
28 pv fici A	6667	6977	5333	5217	9362	4390	9362	9362	5333	4889
29 pv fici B	5789	6154	4390	.3810	.5116	8649	5116	5116	6829	6341
31 pv glycines A	.5366	.5714	1 000	8000	.5652	4000	5652	.5652	5455	5455
34 pv incanae	5366	.5714	5455	.5333	.5652	6000	5652	.5652	1 000	9545
35 pv maculifoliigardeniae	8947	9231	5854	.5714	6977	5405	6977	6977	5854	5366
36 pv malvacearum	.5500	5854	9302	7727	5778	4103	5778	5778	5116	5116
37 pv manihotis		9231	5366	5714	6977	5405	6977	6977	5366	4878
40 pv phaseoli A			5714	6047	7273	5789	7273	7273	5714	5238
41 pv phaseoli B				8000	5652	4000	5652	5652	5455	5455
42 pv phaseoli "fuscans				—	5532	3415	5532	5532	5333	5333
43 pv physalidicola						4762	1 000	1 000	5652	5217
44 pv poinsettiicola B							4762	4762	6000	.5500
45 pv poinsettiicola A								1 000	5652	.5217
46 pv pruni									5652	.5217
47 pv raphani A										9545
48 pv raphani B										

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Table D-2—*Continued*

Species/Pathovar	Species/Pathovar								
	50	51	52	53	54	55	56	58	59	61
1 pv alfalfae	8696	8511	8696	3784	8000	6190	4651	.8444	4889	2381
2 pv alfalfae	8696	8511	8696	3784	8000	6190	4651	8444	4889	.2381
3 pv armoraciae	.5217	.5106	5217	5405	.5333	5238	5116	8444	5778	.5238
4 pv begoniae A	7442	7727	7442	5294	7619	9231	5500	.6667	6190	.5128
5 pv begoniae A	7442	7727	7442	5294	7619	9231	.5500	6667	6190	5128
6 pv begoniae B	7442	7727	7442	5294	7619	9231	5500	6667	6190	5128
7 pv bilvae	5957	6250	5957	5263	6087	6047	8182	5217	1 000	4186
8 pv campestris	5652	5532	5652	5946	.5778	5714	5116	4889	5778	5714
11 pv citri A	5217	5532	5217	4865	5333	5714	8837	4444	8444	4762
12 pv citri B	5217	5532	5217	4865	.5333	.5714	7907	4444	9333	4286
13 pv citri C	5217	5532	5217	4865	5333	5714	7907	4444	9333	4286
14 pv citrumelo	9362	9167	9362	4211	8696	6512	5000	7826	.5217	.2791
15 pv citrumelo	1 000	9796	1 000	5128	9362	7273	.5778	8085	.5957	.3636
16 pv citrumelo	1 000	9796	1 000	5128	9362	7273	.5778	8085	5957	.3636
17 pv citrumelo	9362	9167	9362	4737	8696	6977	5455	8696	5652	.3256
18 pv citrumelo	9362	9167	9362	4211	8696	6512	.5000	.7826	5217	2791
19 pv citrumelo	8261	8085	8261	3784	7556	6190	4186	8000	4889	2857
20 pv citrumelo	1 000	9796	1 000	5128	9362	7273	.5778	8085	5957	3636
21 pv citrumelo	8696	8511	8696	3784	8000	6190	4651	8444	4889	2381
22 pv citrumelo	9583	9388	9583	5128	8936	7273	5333	.7660	.5957	4091
23 pv dieffenbachiae A	7273	7556	7273	.5714	7907	1 000	5854	6977	6047	5000
24 pv dieffenbachiae A	7273	7556	7273	.5714	7907	1 000	5854	6977	6047	5000

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Table D-2—*Continued*

Species/Pathovar	Species/Pathovar									
	50	51	52	53	54	55	56	58	59	61
25 pv dieffenbachiae B	8511	8333	8511	5263	7826	6047	5455	6522	5217	2791
26 pv fici A	1 000	9796	1 000	5128	9362	7273	5778	.8085	5957	3636
27 pv fici A	9362	9167	9362	4211	8696	6512	5000	7826	5217	2791
28 pv fici A	9362	9167	9362	4737	8696	6977	5455	8696	5652	3256
29 pv fici B	5116	5000	.5116	5294	5238	6154	4500	4286	4286	6154
31 pv glycines A	5652	5532	.5652	5405	.5778	.5714	9302	4889	8444	4762
34 pv incanae	5652	.5532	5652	5946	.5778	.5714	5116	4889	.5778	.5714
35 pv maculifoliigardeniae	6977	7273	6977	5882	7619	9231	6000	6667	6190	5641
36 pv malvacearum	5778	5652	5778	5556	5909	5854	1 000	5000	.8182	4390
37 pv manihotis	6977	7273	.6977	5294	7619	9231	5500	6667	5714	5128
40 pv phaseoli A	7273	7556	7273	5714	7907	1 000	5854	6977	6047	5000
41 pv phaseoli B	5652	5532	5652	5405	5778	5714	9302	4889	.8444	.4762
42 pv phaseoli "fuscans"	5532	5833	5532	4737	5652	6047	7727	4783	9565	4186
43 pv physalidicola	1 000	9796	1 000	5128	9362	7273	.5778	8085	5957	3636
44 pv poinsettiicola B	4762	4651	4762	4242	4878	.5789	4103	4390	3902	.6316
45 pv poinsettiicola A	1 000	9796	1 000	5128	9362	7273	5778	8085	5957	3636
46 pv pruni	1 000	9796	1 000	5128	9362	7273	5778	8085	5957	3636
47 pv raphani A	5652	5532	5652	5946	5778	5714	5116	4889	5778	5714
48 pv raphani B	5217	5106	5217	5405	5333	5238	5116	4444	5778	5238

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Table D-2—*Continued*

Species/Pathovar	Species/Pathovar									
	50	51	52	53	54	55	56	58	59	61
50 pv vesicatoria A		9796	1 000	5128	9362	7273	5778	8085	5957	3636
51 pv vesicatoria A			9796	5000	9167	7556	5652	7917	6250	4000
52 pv vesicatoria A				5128	9362	7273	5778	8085	5957	3636
53 pv vesicatoria B					5263	5714	5556	4211	5263	4000
54 pv vignicola						7907	5909	8696	6087	3721
55 pv vitians A							5854	6977	6047	5000
56 pv vitians B								5000	8182	4390
58 *X campestris* X198									5217	2791
59 *X. campestris* XCF										4186
61 *X fragariae*										

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BIOGRAPHICAL SKETCH

Rui Pereira Leite, Jr., was born in São Pedro, São Paulo, Brazil on May 26, 1957, to Ruy Pereira Leite and Daysy Fortes Pereira Leite. He attended Rafael de Moura Campos Elementary and Instituto de Educação Cardoso de Almeida High School, Botucatu, São Paulo, where he graduated in 1974. In 1975, Rui enrolled at the Faculdade de Ciências Agronômicas of the Universidade Paulista Júlio de Mesquita Filho - UNESP, Botucatu, São Paulo, where he majored in agriculture and took courses in plant pathology from Dr. Chukichi Kurozawa. Rui graduated with a B.S. from Universidade Paulista Julio de Mesquita Filho - UNESP in 1978. After graduating, Rui took the position of plant pathologist in the Department of Plant Pathology at the Fundação Instituto Agronômico do Paraná - IAPAR, Londrina, Paraná. At IAPAR, Rui carried out research on characterization and control of bacterial diseases of fruit crops. In August 1984, Rui got a study leave and enrolled in the graduate program of the Department of Plant Pathology at the University of Wisconsin, Madison. In Madison, Rui worked with Dr. Doug Rouse on the ecology of *Pseudomonas syringae* pv. syringae that causes the bacterial brown spot on snap beans. Rui graduated with a M.S. degree in plant pathology from the University of Wisconsin in 1986 and returned to his position in the Department of Plant Pathology at IAPAR. In August 1990, Rui enrolled in the graduate program at the University of Florida, Department of Plant Pathology, with Dr. Robert Stall as advisor. Rui was married in 1980 to Claudia Stalzer, and they have two sons, Ruy and Rafael.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy

Robert E. Stall

Robert E. Stall, Chair
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